Characterization of the Relative Contributions of Lin28 Paralogs Lin28a and Lin28b to the miRNA Profile and the miRNA Biogenesis Machinery in Brain

by

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Abstract

Selective control over gene expression is crucial to the brain's ability to learn and form new memories. Assemblies of synapses must be able to modulate their connectivity in a rapid time scale in response to outside stimuli and require fast changes in the pools of expressed proteins to accomplish this. Due to the time required for transcription mediated changes in gene expression, post-transcriptional control over gene expression has magnified importance in neuronal cell types. Previous work in our lab has discovered that Lin28a is an essential mediator of specificity in the post-transcriptional regulation of gene expression in response to BDNF. Lin28 is an evolutionarily conserved RNA binding protein that plays an integral part in pluripotency and differentiation.

In vertebrates, there are two Lin28 paralogs (Lin28a and Lin28b) which share 74.9% of homology. Both paralogs regulate Let-7 miRNAs, but differ in subcellular localization and at which step in miRNA biogenesis this regulation occurs. Given these differences between the paralogs and the lack of their characterization in differentiated cells, it was unknown how functionally redundant they might be in the mammalian brain.

My thesis work focused on characterizing the relative contributions of the two Lin28 paralogs on the miRNA profile and the miRNA biogenesis machinery in the murine brain. I used an approach designed to postnatally knock out each paralog independently in a brain-restricted manner, with the aim of avoiding previously reported developmental phenotypes associated with Lin28 loss. Surprisingly, results from the first part of my thesis work suggest a possible continued postnatal role for the Lin28 paralogs in brain growth. For this purpose, I used conditional knock out mouse lines in which the

functionally essential second exons of Lin28a and Lin28b were flanked by Lox P sites. Cre-mediated recombination at the Lin28a or Lin28b locus using postnatal day 0 (P0) introduction of a viral vector harboring a ubiquitously active promoter resulted in reduced body and fore- and midbrain size in P13 mice. Additionally, a previously reported prenatal role in neurogliogenesis may be preserved postnatally, but requires further confirmation to rule out alternative explanations. Reduction in levels of Lin28a and Lin28b also reduced levels of the miRNA biogenesis protein Dicer, and overall counts of mature miRNAs as assayed in the NanoString platform. Results from miRNA profiling will require further experiments for confirmation, but suggest the possible addition of mir-204, a highly expressed neuron-enriched miRNA, to the list of Lin28 regulated miRNAs. In order to determine whether the smaller brain size and potential lethality observed with postnatal Lin28a deficiency could be due to induction of apoptosis after loss of Lin28 in neurons, the second part of my thesis investigated the effects of postnatal neuron specific loss of Lin28a in the murine hippocampus. The transgenic mouse line expressing inducible Cre recombinase (Cre^{ERT2}) under control of a forebrain neuron-selective promoter (CaMKIIα) was crossed to the conditional Lin28a line to make a new line (CaMKII/$Lin28a^{f/f}$). After inducing Lin28a loss, I found no difference in the levels of apoptosis at the time points used in the first part of my thesis. Further studies could determine whether induction of apoptosis is effected after prolonged loss of Lin28a.

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Chapter I: Introduction:

Regulation of Gene Expression in Brain/Neuroplasticity

The formation of long term memories requires accurate control over changes in gene expression at the level of transcription and translation (Sutton and Schuman 2005; Kida et al. 2002; Mayford et al. 1996). Synaptic plasticity is the strengthening and weakening of synapses and long-term synaptic plasticity is studied as a cellular correlate of learning and memory (Bliss and Collingridge 1993). Much like learning and memory, inhibition of gene expression both at the level of transcription or translation has been shown to prevent enduring forms of synaptic plasticity. The hippocampus is seen as a major center in the brain for learning and memory, and is thus the focus of many experiments interrogating the mechanisms of synaptic plasticity and gene expression (Tyler et al. 2002; Tronel, Milekic, and Alberini 2005). Each neuron can have many thousands of synapses, and thus the regulation of the local suite of proteins at every synapse is key for differential regulation of synaptic strength. While this could theoretically be achieved through highly regulated spatiotemporal trafficking of proteins, it has been shown to be at least partially achieved through local protein synthesis. This makes post-transcriptional regulation of gene expression especially important in neurons. Regulation of local protein synthesis is necessary for lasting activity dependent modification of synaptic transmission. Both Long Term Potentiation (LTP) as well as Long Term Depression (LTD) have been observed to include local translation-dependent phases (Pfeiffer and Huber 2006). While both types of plasticity have early phases that

are independent of changes in gene expression, later phases require new translation, and/or new transcription (Huber, Kayser, and Bear 2000; Pfeiffer and Huber 2006).

BDNF is a Critical Factor for Neuroplasticity

Brain Derived Neurotrophic Factor (BDNF) is a growth factor with significant importance in a diverse set of neuronal functions. It was originally characterized as the second neurotrophic factor after Nerve Growth Factor (NGF) in 1982 (Barde, Edgar, and Thoenen 1982). It is found throughout the central nervous system (CNS) and parts of the peripheral nervous system (PNS), but its highest expression levels are in hippocampus (Hofer et al. 1990). Apart from being important for differentiation and survival of neurons as a trophic factor, BDNF has been shown to be critical for hippocampal LTP, and later the persistence of long term memories formed in the hippocampus (Bekinschtein et al. 2008; Korte et al. 1995). BDNF mRNA is itself trafficked and targeted to local translation in dendrites and spines as well as in axons and pre-synaptic compartments. It is translated in an initial pro-BDNF form, before further processing into mature BDNF which is recognized by the Tyrosine receptor kinase B (TrkB) receptor (Kuczewski, Porcher, and Gaiarsa 2010). TrkB is a member of the receptor tyrosine kinase family, and activates the phosphatidylinositol-3-kinase (PI3K), mammalian target of rapamycin (mTOR), extracellular signal-regulated kinase (ERK), and phospholipase c gamma (PLC-γ) pathways after ligand binding. BDNF has been shown to be released in an activity dependent manner in both the PNS and CNS, and to stimulation frequency encoding release (Balkowiec and Katz 2000; Balkowiec and Katz 2002). Secretion of BDNF can occur from either the dendritic or axonal compartment, and is calcium signaling-dependent (Kohara, Kitamura, and Morishima 2001; Kolarow, Brigadski, and

Lessmann 2007). In hippocampus, excitatory neurotransmitter release is enhanced by BDNF. Neuronal morphology also changes, with BDNF eliciting growth at both the level of dendritic arbor as well as spine size and density (Tyler et al. 2002).

Roles in Disease

BDNF expression has been found to be expressed at lower levels in schizophrenic patients in a meta-analysis across multiple studies (Green et al. 2011). Deregulation of BDNF expression has also been found in mouse models of Rett syndrome, a neurodevelopmental disorder that affects 1 in 15,000 women (Li and Pozzo-Miller 2014). Exogenous expression of BDNF has rescued some Rett-like phenotypes in these mouse models. BDNF has been investigated for a variety of potential therapeutic applications due to its trophic and pro-survival functions in the brain. Pre-clinical studies have shown beneficial effects in models of Alzheimer's, stroke, depression, and other disorders. Huntingtin, the protein mutated in Huntington Disease, has been mechanistically linked to driving BDNF transcription. The mutant form, containing CAG repeat expansion, has been shown to lead to lower BDNF levels (Zuccato and Cattaneo 2009). A relatively common polymorphism, the Val66Met substitution in the pro-BDNF sequence, is associated with deficits in episodic memory and hippocampal volume (Nagahara and Tuszynski 2011). Though the BDNF signaling pathway itself has not been found to be the main cause of these disease states, dysregulation of the BDNF pathway has been implicated in the pathology of Alzheimer's (Jerónimo-Santos et al. 2014).

Regulation of Gene Expression by BDNF

BDNF effects on gene expression have been shown to work through the PI3K, mTOR, ERK, PLC-y pathways. While both transcription and translation are regulated

downstream of TrkB activation, BDNF's effects on translation hold the most promise for the study of many synaptic processes due to the short time frames involved, while the transcriptional response may be important for many trophic requirements (Chang, Poser, and Xia 2004). BDNF induced transcription factors include cAMP response element binding protein (CREB), serum response factor (SRF), and nuclear factor kappa b (NF-κB) (Kalita et al. 2006; Kajiya et al. 2009; Riccio et al. 2006). The enhancement of global translation by BDNF, detected by radiolabel incorporation, is mediated by the activation of both translation initiation as well as elongation factors (Takei et al. 2001; Kanhema et al. 2006).

BDNF has been shown to increase the translation of specific transcripts in multiple model systems, and to affect synaptic plasticity both pre- and post-synaptically. The BDNF mediated increase in translation is highly specific. After stimulation by BDNF, only 4% of transcribed mRNAs were increased in translation. Among those mRNAs observed to undergo selective enhanced translation by BDNF treatment, were many synaptic proteins such as calcium/calmodulin dependent kinase II alpha (CaMKIIα) and Arc (Schratt 2004; Yin, Edelman, and Vanderklish 2002; Huang et al. 2012). The pool of synaptic transcripts encompasses a wide variety of functional pathways including the transcripts necessary for synaptic pruning or long term depression. In order for increased translation to encode a growth response, specificity of the signal for transcripts of pro-growth proteins is required. A global upregulation of all transcripts would carry not only a high metabolic cost, but also dilute or possibly reverse a pro-growth signal. Subsequent high-throughput studies confirmed the finding of specific pools of transcripts undergoing increased translation in response to BDNF (Liao

et al. 2008; Manadas et al. 2009). These findings included both proteins reduced in response to BDNF as well as divergences between mRNA and protein levels, suggesting the importance of a post-transcriptional component to the BDNF response. Transcript sequestration in RNA processing bodies (P-bodies) and binding by RNA binding proteins or miRNAs has also been well documented in response to BDNF (Santos, Comprido, and Duarte 2010; Huang et al. 2012). A BDNF responsive RNA binding protein complex, cytoplasmic FMR1-interacting protein 1 – fragile X mental retardation protein (CYFIP1-FMRP), has been observed in these granules as well (Zheng, Chen, and Shyu 2011).

Post-transcriptional regulation of gene expression is a major driver of protein level changes

Studies of regulation of gene expression have often found lack of concordance between transcript and protein levels, pointing to the importance of post transcriptional regulation of gene expression (Tian et al. 2004; Griffin et al. 2002). Recent studies have shown that mRNA levels become more decoupled from the levels of their coded proteins in human brain over the course of development, and that the majority of discordant mRNAs harbor microRNA binding sites, making them likely targets of post-transcriptional regulation (Wei et al. 2015).

Cells use many modes of action to induce changes in gene expression post-transcriptionally. Transcripts can be bound by RNA binding proteins (RBPs) which can enhance or inhibit translation, and target mRNAs to subcellular domains to spatially restrict expression or initiate degradation. Protein levels can also be modulated post-translationally, with changes in the rate of targeting to the proteasome or lysosome.

Finally, and of most interest to our lab, transcripts can be regulated by small ~21-23 nucleotide long non-coding RNAs called microRNAs (miRNAs).

MiRNA Mediated Regulation of Gene Expression

Biogenesis and mode of action

The first miRNA, lin-4 (mir-125) was discovered in *C. elegans* in 1993 as a regulator of the heterochronic gene lin-14, but was thought to be an isolated case (Rosalind C Lee 1993). It wasn't until years later that the first let-7 miRNA was discovered (Reinhart et al. 2000; Hutvágner et al. 2001). Let-7 was found to be evolutionarily conserved across species and soon miRNAs were recognized as distinct class of small regulatory RNAs, after the elucidation of the siRNA pathway (R C Lee and Ambros 2001; Lau et al. 2001; Lagos-Quintana et al. 2001).

MiRNAs inhibit their targets through base-pairing to complementary sections of mRNAs. These target sites or miRNA Recognition Elements (MREs), which need only be ~6 nucleotides long to be effective predictors of miRNA regulation, are found predominantly in the 3'UTR of mRNAs but can be distributed throughout the transcript. The sequence that most strongly predicts regulation of transcripts is called the seed sequence, and is located at bases 2 to 8 on the 5' end of the mature miRNA. In this manner, a single miRNA species can regulate hundreds of different transcripts, and each transcript can be combinatorically regulated by many different miRNAs. Over 60% of expressed human mRNAs contain evolutionarily conserved miRNA binding sites in their 3'UTRs (Friedman et al. 2009).

The canonical miRNA expression pathway begins by RNA Polymerase II transcribing the primary transcript (pri-miRNA), which can contain multiple different miRNA hairpins in its secondary structure. The pri-miRNA is then processed by the microprocessor complex, containing DiGeorge Syndrome critical region 8 (DGCR8) and Drosha, an RNase III enzyme that produces the precursor miRNA (pre-miRNA). The pre-miRNA has a characteristic 3' two nucleotide overhang in its stem-loop which is fundamental to its further recognition and processing. Some non-canonical miRNAs, called mirtrons, are encoded in introns or exons of protein coding genes and are processed by the splicing machinery rather and the microprocessor. Pre-miRNAs are then exported to the cytoplasm by Exportin-5 for further processing by Dicer, another RNAse III enzyme. Dicer cleaves the hairpin's loop and stem, producing imperfect RNA duplex containing a mature "guide" miRNA and a usually rapidly turned over "passenger" strand. In some cases, the both strands can go on to regulate target mRNAs (Okamura, Chung, and Lai 2008). Mature miRNAs are loaded into the central protein of the RNA Induced Silencing Complex (RISC), Argonaute (Ago).

MiRNAs can either buffer stochastic variability in gene expression networks by fine tuning protein levels, or create large regulatory impact (Ebert and Sharp 2012). Examples of both binary switch type regulation as well as tuning or dampening protein levels are well established (Bartel 2009). In a switch interaction, the miRNAs regulation acts to repress target protein expression below a functional threshold, either as a failsafe for stochastic noise in other regulatory mechanisms or as a primary regulator. In a tuning interaction, the miRNA instead modulates protein levels to a more optimal expression level for the necessary cell state, but not below functional levels.

Over longer time periods, miRNA repression is thought to act primarily through destabilization of target transcripts, though in short term regulation sequestration and lowered translational efficiency has been observed (H. Guo et al. 2010). After conflicting reports of miRNA mediated regulation of protein levels acting in mRNA level dependent or independent modes in both targeted as well as global studies, kinetic analysis showed miRNAs are able to repress translation for hours prior to eventual deadenylation and decay of targeted mRNAs (Djuranovic, Nahvi, and Green 2012). Thus miRNAs present the cell with a diverse toolkit that can have a wide range of effect sizes on the regulation of gene expression.

MiRNAs in Brain and Brain Development

Over half of known miRNAs are expressed in brain (Kosik and Krichevsky 2005). Genes are often expressed with their longest 3' UTR isoform in brain compared with other tissues, and these longer UTRs may help coordinate the post-transcriptional regulation of entire signaling pathways, especially ion channels and transporters (Wehrspaun, Ponting, and Marques 2014). Many of these elongated 3' UTRs are specifically enriched for binding sites of miRNAs more highly expressed in brain (Miura et al. 2013). Sequencing of transcripts that immunoprecipitated with neuronally expressed exogenous Ago2-GFP in hippocampus showed more than two thousand significantly enriched in RISC binding (Malmevik et al. 2015).

MiRNAs are expressed in brain to varying degrees of specificity, from brain region and cell type differences, to subcellular compartment specificities (O'Carroll and Schaefer 2013). MiRNA precursors are found enriched both in synaptoneurosomes, an ex-vivo preparation containing sealed pre- and postsynaptic compartments, as well as the

axonal compartment, ready to be processed for local post-transcriptional regulation of gene expression (Olde Loohuis et al. 2012).

Some miRNAs, like mir-124, -9, -138, and -128 are expressed in a brain specific manner, while others such as mir-29a, -143, -98, -26a, -30a and the let-7s are highly expressed but not as highly brain specific (Landgraf et al. 2007). The let-7s are among the most highly expressed miRNAs in brain, making up between 20-60% of all miRNAs depending on detection method (Juhila et al. 2011). The let-7s have been shown to regulate many cell cycle genes including CDC25A, CDK6 and cyclin D1, arresting proliferation through their regulation (Büssing, Slack, and Großhans 2008). This control over proliferation is particularly important in an environment with such a diverse mix of post-mitotic and quiescent cell types.

Many brain enriched miRNAs have been reported to play important roles in neuronal processes. Early in development, Sox 2 is targeted by mir-200 in neural progenitor cells and this negative feedback loop leads to differentiation (Peng et al. 2012). Later in development, mir-125b and mir-132 were shown to regulate synaptic structure and function (Edbauer et al. 2010). The maintenance of long lasting changes in spines following *N*-Methyl-D-aspartate-LTD (NMDA-LTD) was found to depend on mir-191 and mir-135b (Hu et al. 2014). Haploinsufficiency of mir-185 was shown to be responsible for some developmental phenotypes of the 22q11.2 microdeletion highly associated with schizophrenia and other cognitive diseases (Xu et al. 2013).

Post-transcriptional Regulation of miRNAs During Biogenesis

Beyond representing a post-transcriptional mode of regulation of gene expression, miRNAs themselves are regulated post-transcriptionally at various steps of the biogenesis pathway. Drosha processing of specific miRNAs is enhanced by P72 helicase binding, and interference from a mutant form of the P53 tumor suppressor attenuates this enhancement (Garibaldi et al. 2016; Suzuki et al. 2009). Dicer processing of miRNA is adjusted by partner protein binding, adjusting the site of pre-miRNA loop cleavage based on binding status. Transactivation response RBP (TRBP) binding modifies mature mir-132 length in this manner (Fukunaga et al. 2012; Rybak-Wolf et al. 2014; Starega-Roslan et al. 2015). TRBP binding has been shown to modulate Dicer cleavage for accurate miRNA expression in other miRNAs as well (Wilson et al. 2015).

Ago2 is targeted by E3 ubiquitin ligase lin-41 (TRIM71), a let-7 target. Lin-41 is an essential gene during embryogenesis but is also specifically and robustly expressed in cells lining the lateral ventricles of postnatal mice (Rybak et al. 2009; Cuevas et al. 2015). There are many additional proteins shown to regulate miRNA biogenesis at different steps in the processing pathway, from transcription to editing of the mature sequence in order to destabilize or change the target specificity. For example, KH-type splicing regulatory protein (KSRP) promotes let-7 biogenesis while hnRNP A1 opposes it and both do so by binding the pre-E loop of the pri-miRNA (Trabucchi et al. 2009; Michlewski and Cáceres 2010). RNA specific adenosine deaminases (ADARs) are able to change the target specificity of miRNAs through adenosine to inosine deaminations (Kawahara and Nishikura 2007). Many of these factors are engaged in unilateral or reciprocal feedback loops with the miRNAs they regulate (Krol, Loedige, and Filipowicz

2010). For example MYB enhances the transcription of mir-15a while itself harboring mir-15a target sites. Alternatively, YAN is regulated by mir-7 but also represses its transcription.

miRNA Biogenesis Proteins in Brain and Brain Development

Beyond individual miRNAs, differences in the levels of miRNA processing proteins result in a wide array of cognitive phenotypes. Cortical pyramidal neuron specific DGCR8 KO reduces interneurons in a non-cell autonomous manner and leads to microcephaly (Hsu et al. 2012). Extending these findings, DGCR8 and DROSHA sequestration by CGG repeat RNA aggregates and subsequently reduced miRNA processing was shown to be responsible for some of the neurodegenerative phenotype of Fragile X-associated tremor/ataxia syndrome (FXTAS) (Sellier et al. 2013). Even heterozygous deletion of DGCR8 reduces neurogenesis in the dentate gyrus and reduces levels of several brain enriched miRNAs as well as Igf2. Expression of exogenous Igf2 rescues many of these phenotypes (Ouchi et al. 2013). Further, brains haploid for DGCR8 have reduced number of cortical neurons and smaller spines, as well as reduced dendritic arbors of hippocampal neurons (Fénelon et al. 2011; Stark et al. 2008). Comparison of homozygous postnatal neuron specific KO of DGCR8 and Dicer revealed microcephaly and lowered survival in both, with the more severe phenotype in Dicer possibly arising due to the importance of DGCR8 independent non-canonical miRNAs, which are still sensitive to Dicer levels (Babiarz et al. 2011).

Postnatally induced excitatory neuron specific Dicer knockout (KO) in adult mice increases some measures of learning and memory but eventually leads to neurodegeneration. Surprisingly, miRNA levels continued reducing for weeks post recombination, suggesting that there could be a large dispersion in the half-lives of neuronal miRNA species (Konopka et al. 2010). Dicer conditional KO in this model causes cell loss in hippocampus but not the arcuate nucleus, raising the possibility of an increased importance for postnatal miRNA regulation in hippocampus. Furthermore, this deletion leads to hyperphagia induced obesity, which can be rescued by mir-103 overexpression. The PI3K-mTOR pathway is hyperactive and plays a causative role in this phenotype (Vinnikov et al. 2014). This obesity is transient and possibly reversed by eventual neurogenesis and synaptic reorganization (Mang et al. 2015). Neuron specific Dicer deletion surprisingly only leads to a ~20% reduction in canonical miRNA levels in whole hippocampal tissue, and even smaller change in let-7 levels, suggesting significant let-7 expression in non-neuronal cell types (Babiarz et al. 2011).

The Lin28/Let-7 Pathway – A conserved regulator of miRNA biogenesis and its Target

Discovery of Lin28

The most well-known regulator of the let-7 family of miRNAs is Lin28. Lin28 was discovered in *C. elegans* as a heterochronic gene regulated by miRNA and implicated in post-transcriptional regulation via its own RNA binding motifs (Moss, Lee, and Ambros 1997). Lin28 was discovered and studied due to the precocious developmental phenotype associated with its deficiency. Worms lacking functional Lin28 developed traits of later developmental stages one larval stage earlier and experienced

related pathologies(Ambros and Horvitz 1984). Lin28 was found to be evolutionarily conserved across multiple species including humans, and consistently downregulated with development (Moss and Tang 2003). Many tissues were found to express Lin28 during organogenesis in mouse, and it was mostly absent in adult tissues with the notable exception of skeletal muscle, cardiac muscle, and some GI epithelial cells (D. H. Yang and Moss 2003). This evolutionary conservation extends to Lin28's function, with both its regulation of developmental timing as well as the targets sites in its own 3' UTR being preserved.

Let-7 Dependent and Independent Actions of Lin28

Lin28 was already known to be targeted by mir-125 and let-7, and hypothesized to inhibit let-7s, but the mode of action was still unknown (Slack and Ruvkun 1997). Years later, in human cell line experiments, it was discovered that both Lin28a and Lin28b, two mammalian paralogs of Lin28, bind let-7 precursors. Lin28a binding leads to the 3' oligouridylation of pre-let-7s by TUT4, a terminal uridyl transferase (TUTase). This modification blocks further processing by Dicer, and leads to eventual degradation (Heo et al. 2009; Heo et al. 2008). TUTases can also promote let-7 biogenesis through monouridylation, adding an additional regulatory fulcrum to the biogenesis pathway (Heo et al. 2012). Both the cold shock domain as well as the zinc knuckles of Lin28 bind GnGAn motifs in let-7 precursors simultaneously (Nam et al. 2011).

It is believed Lin28 paralog Lin28b arose from a duplication event during evolution (Y. Guo et al. 2006; Graf et al. 2013). Lin28b shares a high level of protein sequence identity (74.9% in mice, 73% in humans) in its coding region with Lin28a, with the majority of difference near the C terminus, where Lin28b is longer and contains two

nuclear localization sequences (Fig1.1a). These sequences are functional, and give rise to the divergent subcellular localization of the paralogs, with Lin28a being found primarily in the cytoplasm. Both paralogs retain the evolutionarily conserved cold shock and zinc knuckle RNA binding domains. The lin28b transcript contains a longer 3' UTR than lin28a, harboring more potential MREs. Lin28b prevents the processing of let-7s by sequestering them in the nucleus as primary transcripts (Fig1.1b). Lin28a but not Lin28b overexpressing tumors can be inhibited by depletion of TUT4, implicating a different TUTase or degradation mechanism is recruited by Lin28b (Piskounova et al. 2011). Colon and breast tumors often express one or the other paralog but not both.

Non-let-7 miRNAs, including mir-143, mir-103/107, and mir-9, also contain GGAG motifs in the 3' end of their pre-E loops and are thus bound and regulated by Lin28 (Nowak et al. 2014). This extends the potential sequence space regulated by Lin28 proteins beyond the let-7 seed matched transcripts, and is of special interest because of the elevated expression levels of those miRNAs in brain. Mir-9 has been reported to be functional in the BDNF pathway, acting in a manner that depends on dose and duration. Mir-9 was shown to be downregulated in axons by local application of BDNF over a 2 hour time course, but upregulated in response to a higher dose and a 48 hour time course (Dajas-Bailador et al. 2012).

Beyond miRNAs, Lin28a and Lin28b have been found to bind similar but distinct sets mRNAs, including their own, which also contain GGAG motifs similar to those in the let-7 pre-element. Binding of transcripts primarily enhances translation of targets, and has been reported to affect splicing (Hafner et al. 2013; Wilbert et al. 2012; J. Yang et al. 2015). Lin28 binds IGF2 mRNA and enhances its translation, and can be found localized

to stress granules (Polesskaya et al. 2007). Other RNA granules, P-bodies, have also been shown to harbor Lin28a, where it can modulate in a very localized manner the binding of target mRNAs or the de-repression of let-7 targets (Zheng, Chen, and Shyu 2011). A recent finding that Lin28 binds and unwinds G-quartets in miRNAs and mRNAs suggests possibly an even wider array of regulated sequences, or may serve to eliminate false positive GGAG containing candidates (Elisabeth O'Day et al. 2015).

Lin28 in Vertebrate Stem Cell and Development

Apart from having been detected primarily in stem and progenitor cells initially, Lin28a was found to be one of the core set of four reprogramming factors necessary for generation of induced pluripotent stem cells (iPSCs), and the Lin28a expression level is one of the best indicators of pluripotency (Yu et al. 2007). In a very recent finding that represents the first of its kind, Lin28a was reported to bind promoters and recruit demethylase Tet1 in mouse embryonic stem cells to activate transcription (Zeng et al. 2016). This finding could pose additional questions to previous findings that showed Lin28 was localized to the Nucleolar Precursor Body in embryos pre-implantation and that it was essential for nucleologenesis (Vogt et al. 2012). During further development, the Lin28 paralogs remain highly expressed in germ line tissues and placenta. Overexpression of Lin28 promotes formation of primordial germ cells, and knockdown disrupts their development (West et al. 2009). While c-Myc and NF-kB have been shown to induce Lin28B transcription, not much more is known of which transcription factors control the Lin28 paralogs expression (Shyh-Chang and Daley 2013). Constitutive Lin28a KO is perinatally lethal, while constitutive Lin28b KO exhibits sex specific growth defects. Double KO is lethal during early embryonic development. Lin28a and

Lin28b may have shifted developmental importance because later embryonic KO of Lin28b but not Lin28a phenocopies constitutive KO (Shinoda et al. 2013).

One of the first studies to show that Lin28a prevents let-7 processing also showed that those two components along with mir-125 complete an autoregulatory loop that controls neural stem cell development. While not a member of the regulatory loop, pre-mir-128 processing was shown to be similarly prevented by Lin28a (Rybak et al. 2008). Lin28a and Lin28b have been shown to affect cell fate determination in neural lineages, with overexpression of either Lin28 paralog promoting neurogenesis over gliogenesis. This study also further confirmed previous reports of Lin28 strongly inducing IGF2 expression, this time during neuronal differentiation (Balzer et al. 2010). In embryonic studies, Lin28a and Lin28b KOs reduced neural progenitor cell (NPC) number and brain size. Lin28a overexpression increased NPC number and brain mass (M. Yang et al. 2015). These reports showed the Lin28 paralogs were still functionally important in relatively developed tissues.

Lin28/Let-7 Pathway is Dysregulated in Cancer

Considering the fundamental control over growth and proliferation seen in many examples of the importance of the Lin28/Let-7 axis, it should come as no surprise that it would play a role in cancer. Oncogenes k-Ras, c-Myc and Hmga2 are let-7 targets, and thus Let-7s act as tumor suppressors (Kumar et al. 2008; Kumar et al. 2007). Lin28b is upregulated and let-7s downregulated in sympathetic ganglia tumor neuroblastoma, however let-7 independent effects are observed, so possibly another Lin28 target miRNA or mRNA plays a role (Hennchen et al. 2015). Lin28b is also found to be upregulated in pediatric kidney cancer Wilms tumors, and overexpression recapitulates this phenotype,

while let-7 overexpression rescues in the experimental model (Urbach et al. 2014). Interestingly, it was reported that after an initiating signal, a feedback loop comprised of Lin28b, NF-kB and IL6 is able to transform cells and remain active for generations after the initiating signal has been removed (Iliopoulos, Hirsch, and Struhl 2009). This suggests Lin28 has the potential to act as an epigenetic switch, not requiring an enabling mutation prior to transformation. A possible intervention is the upregulation of the exonuclease DIS3, which was shown to selectively degrade Lin28b but not Lin28a mRNA in multiple myeloma cancer cell lines (Segalla et al. 2015).

Lin28a is more highly expressed in higher grade gliomas with poorer clinical prognosis. Furthermore Lin28a overexpression increased tumorigenicity and invasiveness in a mouse model (Mao et al. 2013). Both Lin28a and Lin28b are upregulated in malignant germ cell tumors but not benign teratomas (West et al. 2009). One or both of the Lin28 paralogs were upregulated in ~15% of surveyed tumors, and higher expression was again associated with worse clinical prognosis (Viswanathan et al. 2009).

Lin28 roles in Adult Tissues

Given the consequences of chronically elevated Lin28 levels in differentiated tissues, it is not surprising to find that well elucidated examples of Lin28s role in adult tissues are rare, since it is very difficult to detect when nearly silenced. Nevertheless the Lin28/let-7 axis has been discovered to play an important role in glucose metabolism. Skeletal muscle specific KO of Lin28a was shown to lower glucose tolerance in mice, while Lin28 overexpression conferred resistance to high fat diet induced diabetes (Zhu et al. 2011). Lin28a overexpression in adult tissues was also shown to enhance tissue repair in a let-7 and mRNA binding dependent manner, though further studies are needed to

determine what effect if any Lin28 at endogenous levels plays in wound healing (Shyh-Chang et al. 2013).

Previous findings in our lab

Recent work in the Meffert Lab has found a critical role for the Lin28/Let-7 pathway in activity-dependent upregulation of proteins important in synaptic plasticity, such as CaMKIIα and GluA1 (Huang et al. 2012). Lin28A and miRNA biogenesis factors Dicer and TRBP are rapidly induced in a transcription independent manner in response to BDNF. This leads to rapid decrease in mature let-7 levels and increase in the levels of a large majority of the miRNAs that undergo changes in expression. Many synaptic proteins' transcripts contain MREs for miRNAs whose precursors contain GGAG-motifs in the pre-E loop. Translation of these mRNAs can be regulated by Lin28 induction and consequent downregulation of precursor miRNAs containing the GGAG motif. Through this bipartite regulation of miRNA biogenesis, the effects on Dicer and Lin28a were shown to be the crucial mediators of specificity in the regulation of gene expression in response to BDNF. Additionally, these experiments showed Lin28 mediated downregulation of let-7s is necessary for BDNF dependent neurite outgrowth (Huang et al. 2012).

How my doctoral work extends current research.

Previous and ongoing work in the Meffert lab led us to ask whether the relative contributions of the two vertebrate Lin28 paralogs might differ in their regulation of the miRNA biogenesis machinery and miRNA profiles in the brain. In order to study this question while avoiding the extensive previously published embryonic effects of Lin28 deficiency, we used conditional knockout mouse lines to postnatally delete either Lin28

paralog. The approach was to attempt recombination in either a ubiquitous or a neuron specific manner, to test the cell type specificity of the effects of either paralog. Surprisingly, postnatal deletion of the lin28 paralogs still has significant developmental phenotypes, even when recombination is brain-restricted.

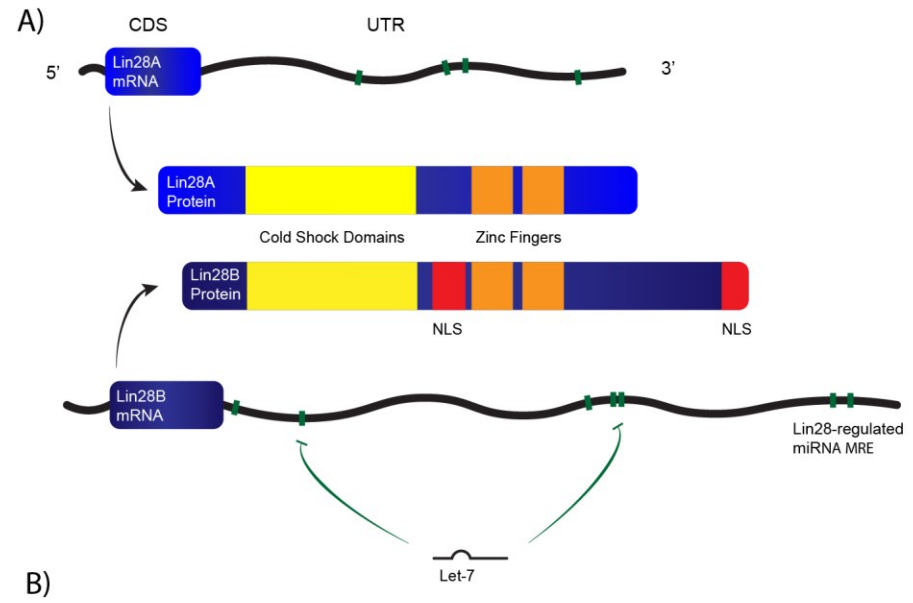
I found significant differences in growth as well as upregulation of glial markers pointing to the possibility of continued influence on cell fate specification or an ongoing trophic requirement for lin28 in the postnatal murine brain. The assayed miRNA profiles showed significant differences in surprising non-let-7 family miRNAs that should be investigated for mechanistic insights into the regulatory relationship leading to their changes.

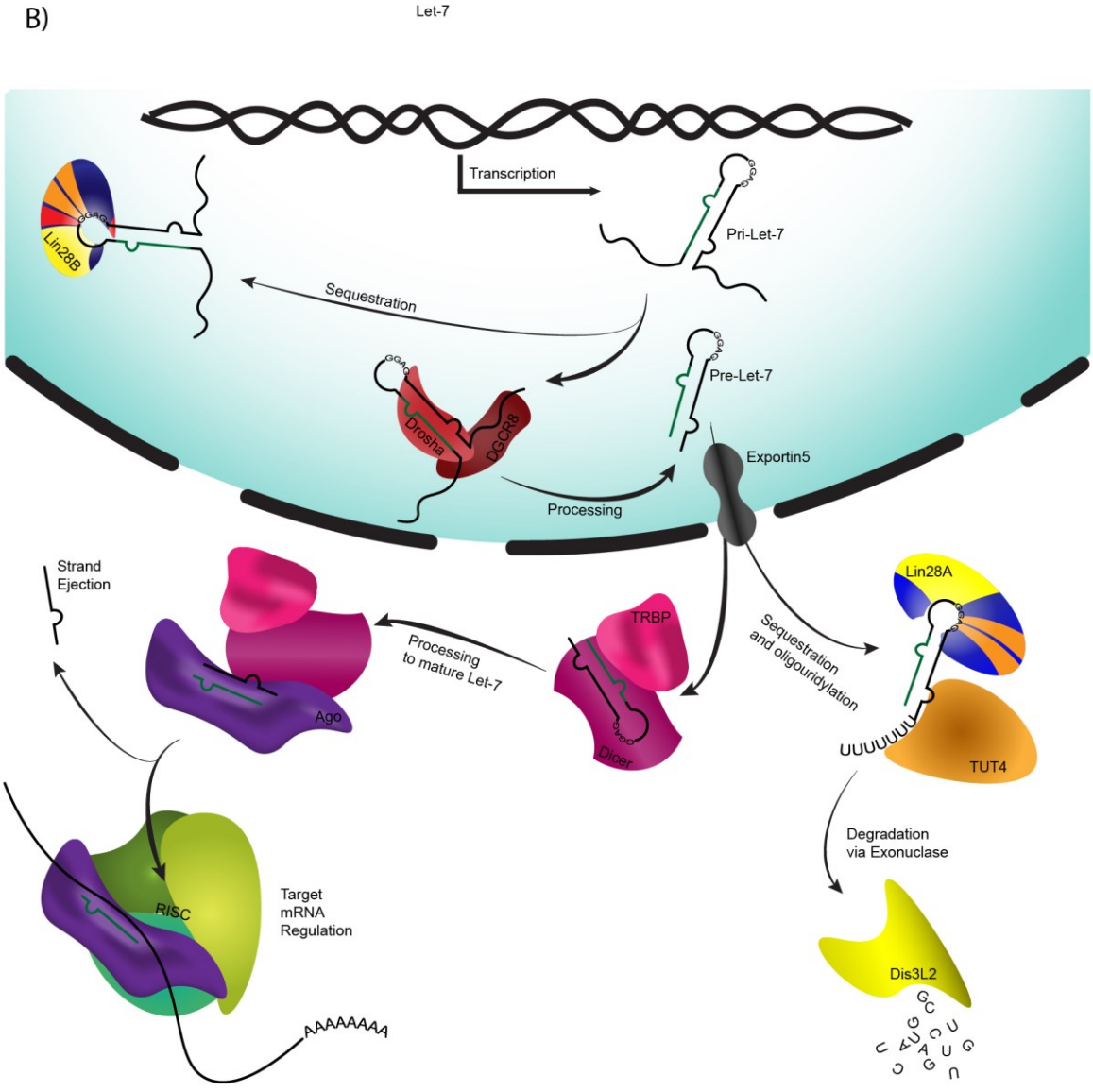
Figures and Legends

Figure 1.1 Structure and Function of the Lin28 Paralogs

A) Schematic view of Lin28a and Lin28b transcripts with MREs of Lin28 regulated miRNAs in their 3' UTRs. B) Diagram of localization and mode of action of the Lin28 paralogs during Let-7 biogenesis.

Figure 1.1


A)
CDS
UTR
5'
3'
Lin28A mRNA
Lin28A Protein
Cold Shock Domains
Zinc Fingers
Lin28B Protein
NLS
NLS
Lin28B mRNA
Lin28-regulated miRNA MRE
Let-7


B)
Transcription
Pri-Let-7
Lin28B
Sequestration
Pre-Let-7
Drosha
DGCR8
Exportin5
Processing
Strand Ejection
Ago
Processing to mature Let-7
TRBP
Dicer
Sequestration and oligouridylation
Lin28A
UUUUUU
TUT4
Degradation via Exonuclase
Dis3L2
RISC
Target mRNA Regulation
AAAAAAAA

Chapter II: Characterization of Post-Natal Ubiquitous Knock Out of the Lin28 Paralogs

Background

The high degree of similarity between the Lin28 paralogs coupled with their differences in action, localization, and developmental timing makes discerning their degree of redundancy in the regulation of miRNAs of interest. The importance of the rapid upregulation Lin28a (within 5 minutes) in the transcription- independent immediate effects of BDNF in hippocampal neurons has been demonstrated previously in our lab (Huang et al. 2012). Lin28b has been shown to be regulated by transcription factors that harbor let-7 sites and thus may be de-repressed by Lin28a induction. Lin28b also downregulates the processing of the Let-7 family of miRNAs, but primarily at the pri- to pre- transition prior to export from the nucleus (Piskounova et al. 2011). This raises the question of whether Lin28b regulation of pri-miRNAs containing GGAG-motifs extends to other miRNAs collocated with them in clusters likely to comprise the same transcript (Figure 2.1a).

Over the course of prolonged activity, Let-7s could be regulated to differing extents at the transcriptional, primary, and secondary processing steps, as well as by changes in the general miRNA biogenesis machinery. By manipulating the levels of the Lin28 paralogs we directly impact the primary and secondary processing of the let-7s, and may additionally have secondary feed-forward of feed-back effects on their transcription and general miRNA biogenesis. Past studies have shown the importance of the ERK pathway in the BDNF response as well as the implicating the phosphorylation of TRBP in the specific downregulation of let-7s in response to ERK pathway stimulation

(Paroo et al. 2009). This, combined with the previous findings in our lab regarding Lin28a's importance in mediating the BDNF response, adds and additional link between the general miRNA biogenesis machinery and Lin28a. The lab has previously shown that Lin28a knockdown in dissociated hippocampal primary neuronal culture does not interfere with the Dicer mediated downregulation of BDNF targets in the first 20 minutes after stimulation, though it remained to be seen whether other components of the miRNA biogenesis pathway might undergo homeostatic responses during long term stimulation or compensator y changes following extended deficiency in either Lin28a or Lin28b. The possibility of an epistatic relationship between the paralogs, with the presence of Lin28a being necessary for the induction of Lin28b, or vice versa, was intriguing. In addition to the transcription factors known to regulate Lin28b, both paralogs harbor multiple MREs of Lin28 regulated miRNAs, and Lin28a is upregulated rapidly in response to BDNF.

The ability to investigate the relative contributions of each paralog to the miRNA profile in hippocampus using a conditional post-natal KO mouse model presented an opportunity to look at the paralogs' respective effects in vivo while avoiding the extensive developmental phenotypes observed in pre-natal deletion (M. Yang et al. 2015; Shinoda et al. 2013). Additionally, substantial effects associated with deficiency of either paralog in postnatal brain would represent additional evidence of functional relevance in a population of predominantly differentiated cells rather than the progenitor and stem cell populations investigated in previous studies.

Results

Brain Restricted Loss of Lin28a or Lin28b has distinct Effects on Levels of miRNA Biogenesis Machinery.

In order to avoid the perinatal lethality of embryonic constitutive KO, we selectively knocked out each Lin28 paralog postnatally in a brain-restricted manner using mouse lines designed to allow for conditional deletion by the Cre recombinase. The mouse lines, from the George Daley lab, have the essential second exon of the respective Lin28 paralog flanked by LoxP sites (Fig 2.1b).

We utilized postnatal day 0 (P0) injections of AAV mediated cre recombinase into the lateral ventricles of mice homozygous for the floxed allele of either paralog. Mice serving as controls were injected at P0 with viral PBS or Adeno Associated Virus (AAV) expressing fluorophore alone (see detailed protocol in Extended Experimental Protocols). Experimental and control mice from the same litters were then harvested and processed on P13. Tissue from a single hippocampus was used to assay DNA, RNA and protein in parallel. The opposite hippocampus was either also processed separately or fixed for later immunohistochemical analysis (Fig 2.1c).

Considering much of the previous data from our lab was from primary neuronal culture, and demonstrated the importance of Lin28a mediated miRNA regulation in neurons, we initially sought to differentiate between ubiquitous loss and neuronal loss of each paralog by utilizing two separate AAV vectors. We selected the AAV CMV Cre-GFP vector for its well established robust ubiquitous expression, and the AAV CaMKII Cre-GFP vector to specifically target neuronal Lin28. While the CMV Cre-GFP

expressed and recombined as expected (Fig 2.2a-c), the CaMKII Cre-GFP exhibited markedly less specificity than expected and recombined almost as widely as the CMV driven vector (Fig. 2.4b).

Though there were possible advantages associated with the potentially developmentally delayed onset of peak CaMKII driven expression compared to CMV, the amount of characterization necessary to test whether the promoter does lose specificity and generate ubiquitous Cre expression and recombination moved us to select CMV as a previously characterized ubiquitous driver of cre expression and to use a transgenic mouse line (covered in Chapter 3) to develop a neuron specific model of Cre recombination.

Immunoblot of hippocampal lysates from P13 mice showed robust reduction in Lin28a levels, with average detected Lin28a at 37 kD being 29% +/- 4% in Lin28a$^{fl/fl}$ mice injected with CMV Cre-GFP compared with those injected with GFP only (n=9 for both conditions from 3 independent yoked experiments $p=2.5x10^{-4}$). Lin28b$^{fl/fl}$ mice showed similarly reduced levels of Lin28b in response to CMV-Cre-GFP (28% +/- 6%, n=9 and 8 from 3 independent experiments, $p=4.97*10^{-5}$) when compared to fluorophore injected controls. Bl6/J control mice lacking the conditional allele and injected with the same viral titer ($1x10^{13}$ genome copies (GC)/ml) showed no significant difference in Lin28a or Lin28b protein levels (Figure 2.4d).

Conditional deficiency of Lin28a was also observed to result in significant decreases in Dicer (55% +/- 7% p=0.038) protein levels but levels of Dicer partner protein TRBP were not significantly altered (Fig2.2c, f, h). Dicer levels were also

significantly lower (40% +/- 11% p=0.04) in Lin28b$^{fl/fl}$ mice injected with CMV-Cre. Additionally, KO of Lin28a led to significant reduction in baseline Lin28b levels (78% +/- 7% p=0.048) while Lin28b KO had no effect on Lin28a levels (Fig2.2d). A decrease in the general miRNA biogenesis machinery coupled with a de-repression of Let-7s is supported by qRT PCR of candidate miRNAs (Fig2.2e, i). Control miRNA mir-132, used as representative of general biogenesis levels, was significantly decreased in response to deletion of either paralog, while let-7 miRNAs were slightly increased or unchanged.

Postnatal deletion of Lin28 leads to differences in Growth and Survival

Surprisingly, in limited observations it appeared that when Lin28a or Lin28b fl/fl mice P0 injected with CMV Cre-GFP were grown up beyond P14, their survival dropped precipitously compared with mock injected controls (1 of 7 injected survival to P21 compared with 9 of 11 mocks, 7 of 15 and 6 of 7 respectively for P17). Though not sufficient n for a determination of statistical significance using Fisher's exact test, this nonetheless led us to examine whether phenotypes previously thought to be limited to embryonic loss of Lin28 were recapitulated in postnatal KO. Alternative explanations for this death, which have not been completely ruled out, include deleterious effects due the overexpression of Cre recombinase or infection resulting from particular viral injections. Prenatal constitutive deletion of Lin28 has been shown to alter neurogliogenesis, brain size, and body mass (Balzer et al. 2010; M. Yang et al. 2015). Brain-restricted and post-natal KO have not yet been studied for trophic or cell fate specific phenotypes. Mice injected with AAV Cre showed significant differences in brain mass excluding cerebellum (where virus does not display robust expression from P0 LV injections) as well as body mass (Fig2.3a, b). This surprising effect on whole body mass from a brain-

restricted KO could potentially be due to behavioral differences in feeding or differences in metabolic control. However, it is important to note that we have not ruled out general deleterious effects (not specifically related to Lin28 deficiency) due to Cre overexpression or infection as noted earlier. An effect on brain mass was not seen in BL6/J mice injected with the same viral titer of Cre (Figure 2.3b, n= 7, 4, 3), Single hippocampi from injected mice and controls were sliced and imaged to measure gross morphology of hippocampal structures. In Lin28a floxed mice CMV-cre mediated recombination led to significantly smaller hippocampal cross-section. This difference was not due to difference in thickness of the neuronal cell body layers, measured by blinded analysis of Hoechst nuclear staining, and thus suggests effects on neuronal projections and/or glial cells (Fig2.3a).

Ubiquitous Lin28a, but not Lin28b KO results in large increase in GFAP

In order to investigate the effects of ubiquitous recombination of the Lin28 paralogs on glia and neurons we assayed the levels of glial fibrillary acidic protein (GFAP) and β-tubulin class III (β-tubIII). GFAP and β-tubIII are widely used as markers of glia (most often astrocytes) and neurons respectively, though are both detected in non CNS cell types and GFAP is a well known marker of neuronal progenitor cells which remain present in the postnatal brain (Davidoff et al. 2002; Buniatian et al. 1998; De Gendt et al. 2011). Atypical expression has also been documented within the CNS in disease states and during development (Hol et al. 2003; Dráberová et al. 2008). In Lin28a floxed mice receiving the highest doses of AAV CMV Cre-GFP and showing the most extensive protein level reduction of Lin28a via immunoblot, GFAP appeared highly upregulated compared to mock injected controls (n=13 and 14 from 4 independent

experiments) while β-tubIII was slightly but not significantly downregulated (Fig2.4a). The exposures of the GFAP immunoblots were outside the linear detection range of our Western blotting film, and I have not reblotted these membranes using an imaging system with a larger dynamic range that may allow quantitation. For this technical reason, I am unable to accurately quantitate the upregulation of GFAP observed with Cre expression in Lin28a$^{f/f}$ mice seen in these in 4 separate experiments. Interestingly, Lin28b floxed mice did not display the same dramatic change in GFAP with similar Lin28b protein level reductions (expressing 28% +/- 6% of mock). While there was a mild, but not significant, increase in GFAP in Lin28b floxed mice, a similar mild increase was seen in Bl6/J control mice and may be partly an off-target side-effect of cre overexpression (Fig 2.4c, d). Further studies are necessary to fully determine the possible relationship, if any, between Lin28a loss, and GFAP upregulation.

miRNA Profiling confirms Lin28 regulation of previously uninvestigated GGAG miRNA and reveals differences between paralogs.

We next attempted to analyze changes in the profile of mature microRNAs that might result from postnatal brain deficiency of the Lin28 paralogs using the NanoString platform. NanoString is a digital profiling technology that does not use amplification or reverse transcription in its quantitation, and thus lacks some of the common sources of experimental variability associated with other profiling technologies. This requires specific barcoded probes for all examined miRNAs, and thus precludes any new miRNA discovery, or editing detection. Within each NanoString assay are a variety of endogenous and exogenous probes in order to control and normalize for most common sources of assay variance.

In an attempt to minimize potential differences in the relative numbers of glial and neuronal cells as a confounding variable in comparing sample miRNA profiles, I chose to submit samples with lower levels of Lin28a knockdown (46%, 57%, 41% and 47% for a combined 48% +/- 3% of mock protein levels) but for which the GFAP upregulation phenotype was also not present (Fig2.4 b).

There are several caveats of this approach, including: 1) the observed changes in the miRNA profile may be more subtle or undetectable in some instances; 2) recombination frequency is well-known to vary in a cell-type specific manner which means that low levels of recombination are prone to producing a cell-specific bias in the recombined population; 3) there is not companion data for this experiment which allows an assessment of the uniformity of Lin28a deficiency, and 4) samples were collected at different times and not subjected to the same storage and handling. A benefit to the approach is that effects seen across samples from experiments done at different times and robust to possible variations in handling lend additional confidence in the result not being an effect isolated to a single cohort. In this case the 4 mock samples from 4 different experiments (1 from each of the treatment condition experiments) correlate and cluster together, as do Lin28a samples from 2 independent experiments and Lin28b samples from 2 independent experiments (Fig 2.6a-c).

Consistent with the previously discussed findings of reduced miRNA biogenesis factors, NanoString profiling revealed generally lower mature miRNA levels in samples from both Lin28a as well as Lin28b floxed mice treated with CMV cre when compared with mock injected controls (Fig 2.6b). When normalized to assay internal ligation controls and housekeeping genes only, out of the 100 most detected miRNAs 5 miRNAs

in Lin28a$^{fl/fl}$ mice and 26 miRNAs in Lin28b$^{fl/fl}$ had significantly changed levels from the mock controls (at $P<0.05$ in a two sided t-test with Bonferroni multiple hypothesis correction). Out of those 31 significantly differently expressed miRNAs, all of them were downregulated. Out of the 100 miRNAs, only 14 were increased on average in Lin28a$^{fl/fl}$ mice treated with CMV-Cre and only 4 in Lin28b$^{fl/fl}$ CMV-Cre samples. Unlike those decreased on average, none of those increased on average were increased to the point of statistical significance.

In order to analyze the effects of each Lin28 paralog on specific miRNA species relative to the general pool, we thus normalized the profile to the geometric mean of the top 100 detected miRNAs while excluding previously confirmed Lin28 targets to preclude any possible Lin28 mediated regulatory effect biasing the normalization. Because each NanoString codeset only allows for testing 12 (4 n for each condition) samples at a time, and as expected, the effects at baseline with no stimulation are relatively mild, no changes in individual miRNAs reach significance via t-test and Bonferroni correction. Assuming similar variance in subsequent samples, multiple miRNAs would reach significance with 8 n instead of 4. When correlated across all samples, biological replicates correlated more highly with each other than with other treatment groups (Fig.2.6c).

Surprisingly, many of the most upregulated miRNAs in response to Lin28a deletion were non-GGAG containing species. (Fig2.5a). However, interestingly, some of these non-GGAG species contained pseudo-GGAG motifs, some of which had been previously reported but not tested (Heo et al. 2009). The canonical GGAG motif shown to be most efficiently bound by Lin28a is the exact sequence GGAG, and situated exactly

4 nucleotides from the beginning of the 3' strand of the mature miRNA. Deviations in the sequence and the distance to the 3' strand have been shown to be severely detrimental to both binding as well as uridylation (Nam et al. 2011; Heo et al. 2009). However, not every let-7 has the exact sequence GGAG or has it exactly 4 nucleotides from the beginning of the 3' mature strand. Recently, it was shown that other secondary structures in the pre-E loop could inhibit regulation of Let-7s even if they contained the canonical GGAG, and that Let-7s could be regulated even with the canonical GGAG mutated to UGCG (Triboulet, Pirouz, and Gregory 2015).

The most upregulated miRNA in deletion of either paralog was the brain and neuron enriched miRNA mir-204 (Fig2.5a, b). This miRNA was not previously published to be regulated by Lin28, but had previously been recognized to have a putative GGAG motif, though by chance was not selected for further study (Heo et al. 2009). In mir-204, the GGAG sits precisely adjacent to the beginning of the 3' mature miRNA strand, and there is a non-canonical GAAG three nucleotides from the 3' mature strand (incorporating the first G of the GGAG). Similarly, mir-15a, a member of a miRNA family that includes previously confirmed Lin28 target mir-107, has a GGUG directly abutting the 3' mature strand. Mir-15a is also upregulated in both Lin28a and Lin28b KO conditions. In fact, of the previously reported Lin28a targets upregulated in this analysis, only Let-7i has the perfect canonical GGAG sequence. The other let-7s that contain it were slightly downregulated. One other evolutionarily conserved GGAG miRNAs not tested by Heo et al but expressed above background, mir-149, was also slightly downregulated.

In contrast to Lin28a KO, more Let-7 miRNAs were at least slightly upregulated in Lin28b KO (Fig2.5b). As in Lin28a$^{fl/fl}$ no individual miRNAs had large enough regulatory differences to reach statistical significance after multiple hypothesis correction, and would require either additional NanoString n or candidate based RT qPCR follow up experiments. The absolute magnitude of miRNA regulation, both up and down, was also greater in the Lin28b floxed samples. Interestingly collocated miRNAs, which were exclusively downregulated in the Lin28a KO condition, were broadly upregulated in Lin28b KO. The exception was mir-145, which is collocated with mir-143, a Lin28 regulated miRNA not detected above background in these assays. This suggests that there is some regulatory effect of Lin28b binding GGAG pri-miRNAs on the processing of non-GGAG "passengers". When pooled as individual members of two different classes of miRNAs, the mean log2 fold changes of GGAG, pseudo-GGAG, and collocated miRNAs are significantly differently regulated in Lin28b$^{fl/fl}$ than the remaining miRNAs ($p=0.039$). Two other probes that had much higher detection in Lin28b KO animals were mir-1937a/b and mir-1937c. These hairpins have been described as being non-canonical and likely of tRNA origin, with no published functional studies confirming their activity, though it would be interesting to investigate what causes this difference in detection levels (Fig2.5 c).

As expected, when clustered using an unsupervised agglomerative algorithm the independent samples from each condition clustered together, and Lin28a and Lin28b KO samples were more closely clustered in relation to mock. Overall, the Lin28 paralog KOs at baseline had more of their most upregulated miRNAs in common than their most downregulated (Fig2.6A). This would be consistent with many of the upregulated

miRNAs being due to relief of direct regulation, while downregulation of miRNAs could be due to more downstream effects such as switches in transcriptional programs, the possibly differential downregulation of TRBP, or changes in levels of Lin28 mRNA targets and their 3' UTRS.

Figures and Legends

Figure 2.1 Approach taken for conditional brain-restricted KO of Lin28 paralogs

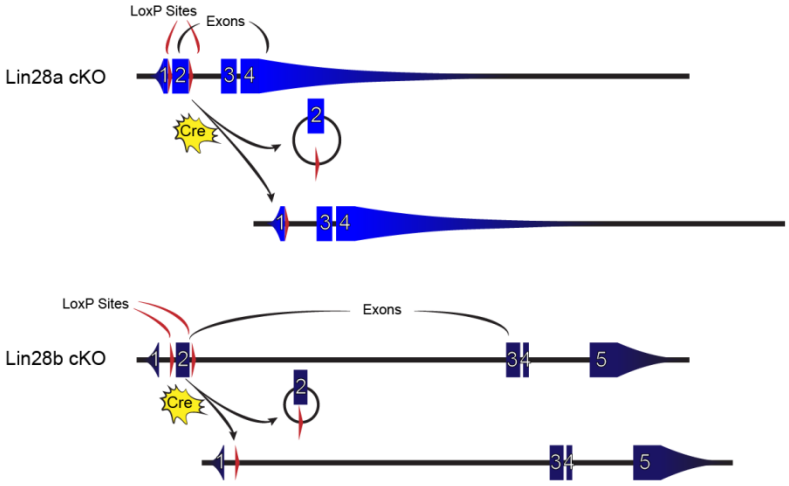
A) Table of confirmed Lin28 regulated miRNAs and the respective miRNAs co-located in putative transcriptional clusters (<10kb). **B)** Genomic organization of Lin28a and Lin28b floxed exons. **C)** Experimental workflow of AAV injection, mouse harvest, and sample preparation for downstream analysis.

A)

Lin28 Regulated miRNAs	Colocated non-GGAG miRNAs
mir-107	
mir-143	mir-145a
mir-9	mir-3093
mir-98	
Let-7a	mir-100
Let-7b	
Let-7c	mir-99a
Let-7d	
Let-7e	mir-99b, mir-125a
Let-7f	
Let-7g	
Let-7i	

Figure 2.1

B)



C)

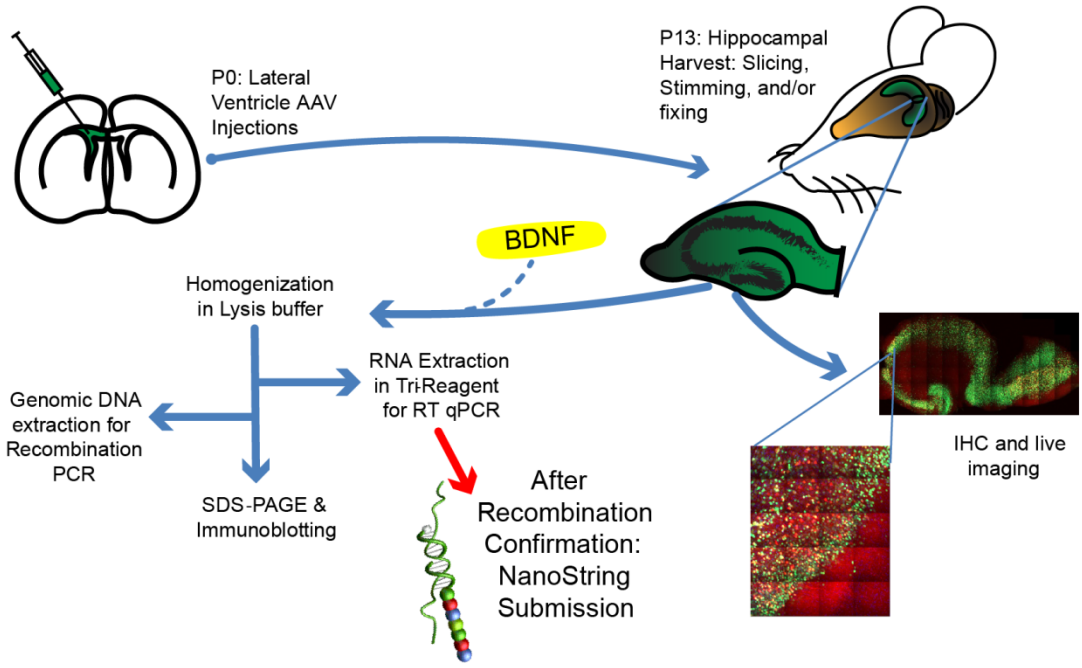


Figure 2.2 Ubiquitous Brain-restricted deletions of Lin28 Paralogs

A) Lin28a protein levels in P13 Lin28a fl/fl whole hippocampal lysates. n=16 and 9 independently injected hippocampi from 8 and 5 mice each. **B)** Semi-quantitative recombination PCR. Primers flanking the entire floxed region amplify the unrecombined and recombined bands with near neutrality, recombined band preference at even input 15%. Faint band below unrecombined band is an unidentified PCR artifact. Ratios represent parts of total template DNA in PCR reaction. **C)** Dicer and TRBP protein level in P13 Lin28a fl/fl whole hippocampal lysates n=13 and 9 independently injected hippocampi from 7 and 5 mice respectively. TRBP: N=4 independently injected hippocampi from 2 mice each. **D)** Lin28B protein levels in Lin28a fl/fl mice n= 4 and 3 independently injected hippocampi from 2 mice each. **E)** qRT PCR of selected miRNAs in Lin28a fl/fl whole hippocampi normalized to sno234. n=13 and 15 independently injected hippocampi from 8 mice each. **F)** Lin28B and TRBP protein in Lin28B fl/fl hippocampus n=9 independently injected hippocampi from 5 mice each. TRBP n=4 independently injected hippocampi from 2 and 3 mice respectively. **G)** Recombination PCR and qRT PCR of Lin28B mRNA normalized to GAPDH in P13 Lin28b fl/fl mice. n=3 independently injected hippocampi from 3 mice each. **H)** Dicer proteins in Lin28B fl/fl whole hippocampal lysates. n=9 independently injected hippocampi from 5 mice each. **I)** qRT PCR of selected miRNAs in Lin28b fl/fl whole hippocampi normalized to sno234. n=7 and 8 independently injected hippocampi from 4 mice each. Statistical significance determined via two tailed Student's t-test * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$. Error bars represent Standard Error of the Mean (SEM).

Figure 2.2

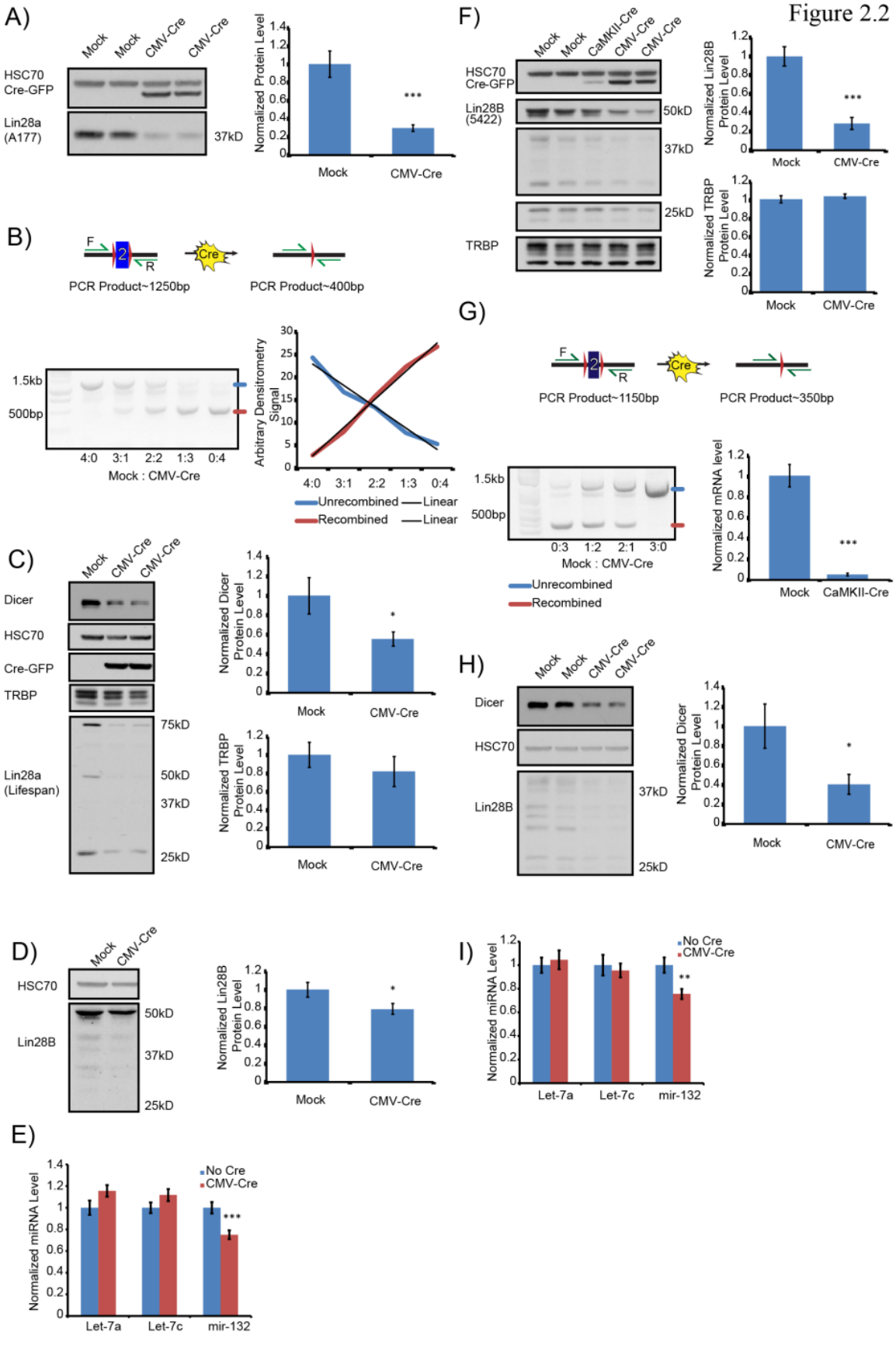

A)
Mock
Mock
CMV-Cre
CMV-Cre
HSC70
Cre-GFP
Lin28a
(A177)
37kD
Normalized Protein Level
Mock
CMV-Cre
B)
F
2
R
Cre
PCR Product~1250bp
PCR Product~400bp
1.5kb
500bp
4:0 3:1 2:2 1:3 0:4
Mock : CMV-Cre
Arbitrary Densitrometry Signal
Unrecombined
Linear
Recombined
Linear
C)
Mock
CMV-Cre
CMV-Cre
Dicer
HSC70
Cre-GFP
TRBP
Lin28a
(Lifespan)
75kD
50kD
37kD
25kD
Normalized Dicer Protein Level
Normalized TRBP Protein Level
D)
Mock
CMV-Cre
HSC70
Lin28B
50kD
37kD
25kD
Normalized Lin28B Protein Level
E)
No Cre
CMV-Cre
Normalized miRNA Level
Let-7a
Let-7c
mir-132
F)
Mock
Mock
CaMKII-Cre
CMV-Cre
CMV-Cre
HSC70
Cre-GFP
Lin28B
(5422)
50kD
37kD
25kD
TRBP
Normalized Lin28B Protein Level
Normalized TRBP Protein Level
G)
F
2
R
Cre
PCR Product~1150bp
PCR Product~350bp
1.5kb
500bp
0:3 1:2 2:1 3:0
Mock : CMV-Cre
Unrecombined
Recombined
Normalized mRNA level
Mock
CaMKII-Cre
H)
Mock
Mock
CMV-Cre
CMV-Cre
Dicer
HSC70
Lin28B
37kD
25kD
Normalized Dicer Protein Level
Mock
CMV-Cre
I)
No Cre
CMV-Cre
Normalized miRNA Level
Let-7a
Let-7c
mir-132

Figure 2.3 Developmental phenotypes of post-natal brain-restricted Lin28 loss

A) Body mass of P13 Lin28a fl/fl pups: n=2, 2 and 4 mice. Fore- and midbrain mass of Lin28a fl/fl pups: n=2, 6 and 6 mice. Hippocampal gross morphology in hippocampal slices from P13 Lin28a fl/fl mice: n=4 and 5 independently injected hippocampi from 4 and 5 mice respectively. **B)** Body mass of P13 Lin28b fl/fl pups: n=4, 2 and 6 mice respectively (Left graph). P13 fore- and mid-brain mass of Lin28b fl/fl and Bl6/J pups: n=2, 5, and 5 mice each for floxed and 7, 4, and 3 respectively for BL6/J mice (Right graph). Measurements were taken blinded to treatment status of the mice. Statistical significance determined via two tailed Student's t-test * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$. Error bars represent SEM.

Figure 2.3

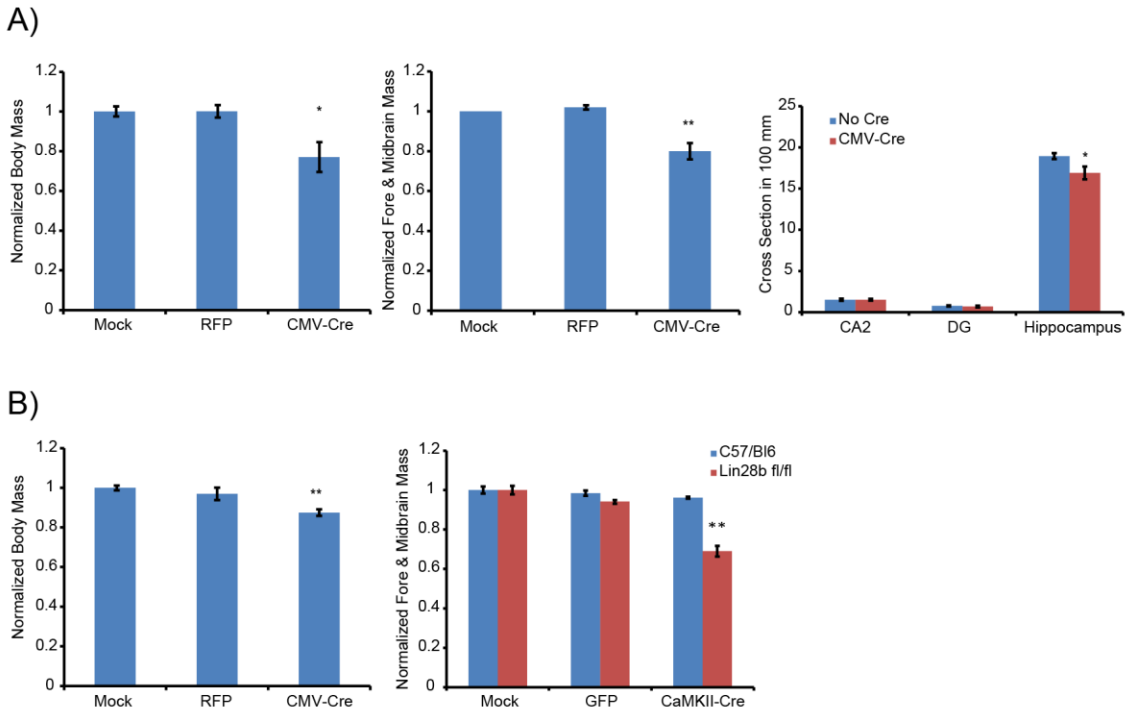

A)
Normalized Body Mass
1.2
1
0.8
0.6
0.4
0.2
0
Mock
RFP
CMV-Cre
*
Normalized Fore & Midbrain Mass
1.2
1
0.8
0.6
0.4
0.2
0
Mock
RFP
CMV-Cre
**
Cross Section in 100 mm
25
20
15
10
5
0
No Cre
CMV-Cre
CA2
DG
Hippocampus
*
B)
Normalized Body Mass
1.2
1
0.8
0.6
0.4
0.2
0
Mock
RFP
CMV-Cre
**
Normalized Fore & Midbrain Mass
1.2
1
0.8
0.6
0.4
0.2
0
Mock
GFP
CaMKII-Cre
C57/Bl6
Lin28b fl/fl
**

Figure 2.4 Extensive deletion of Lin28a, but not Lin28b, results in strongly increased levels of GFAP.

A) Representative immunoblots of GFAP and β-tub III in Lin28a fl/fl KO. For β-tub III quantitation n=10 and 7 independently injected hippocampi from 6 and 5 mice each. **B)** Lin28a protein levels in samples submitted for NanoString analysis. n=2 and n=4 independently injected hippocampi from 2 and 3 mice each. **C)** GFAP protein levels in Lin28b fl/fl hippocampus n=11 independently injected hippocampi from 6 mice each. **D)** Lin28a, Lin28b, Dicer, and GFAP protein levels in P13 C57/BL6 control mice treated with AAV CMV-Cre. n=2 and 4 independently injected hippocampi from 2 and 4 mice each. Results from 2-4 independent experiments for all panels. Statistical significance determined via two tailed Student's t-test * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$. Error bars represent SEM.

Figure 2.4

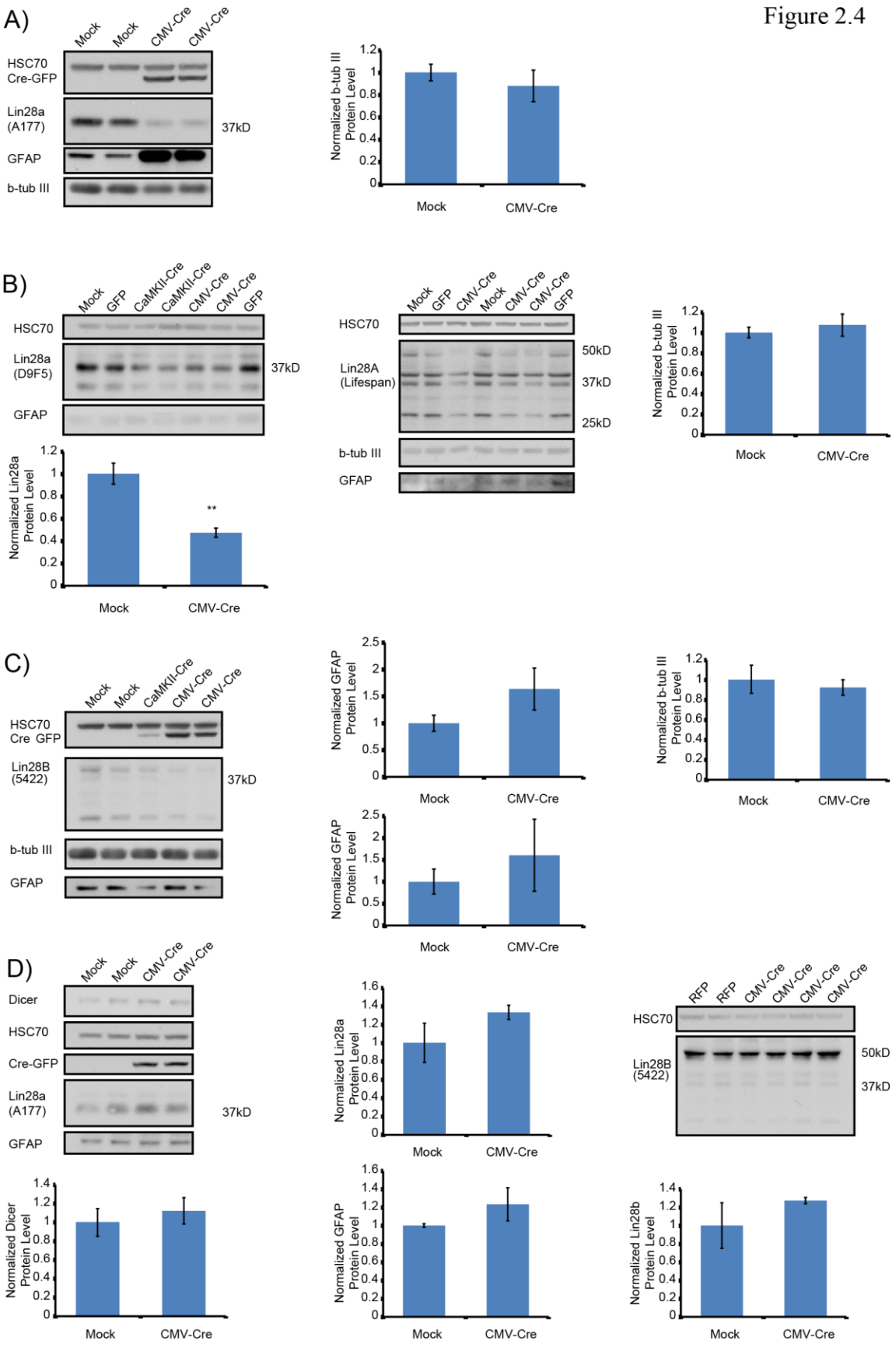
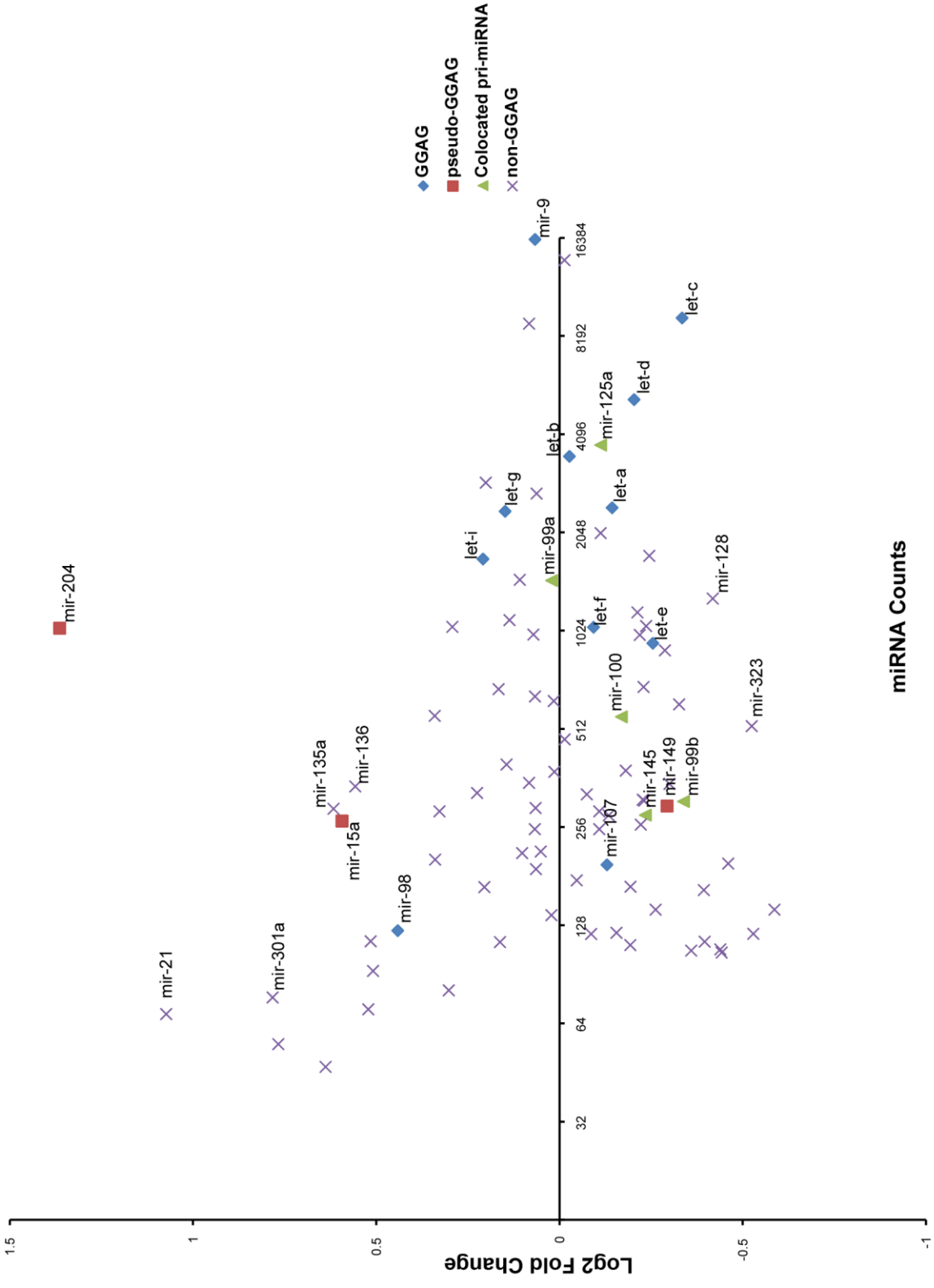

A)
Mock
Mock
CMV-Cre
CMV-Cre
HSC70
Cre-GFP
Lin28a
(A177)
37kD
GFAP
b-tub III
Normalized b-tub III Protein Level
Mock
CMV-Cre
B)
Mock
GFP
CaMKII-Cre
CaMKII-Cre
CMV-Cre
CMV-Cre
GFP
HSC70
Lin28a
(D9F5)
37kD
GFAP
Normalized Lin28a Protein Level
**
Mock
CMV-Cre
Mock
GFP
CMV-Cre
Mock
CMV-Cre
CMV-Cre
GFP
HSC70
Lin28A
(Lifespan)
50kD
37kD
25kD
b-tub III
GFAP
Normalized b-tub III Protein Level
Mock
CMV-Cre
C)
Mock
Mock
CaMKII-Cre
CMV-Cre
CMV-Cre
HSC70
Cre GFP
Lin28B
(5422)
37kD
b-tub III
GFAP
Normalized GFAP Protein Level
Mock
CMV-Cre
Normalized GFAP Protein Level
Mock
CMV-Cre
Normalized b-tub III Protein Level
Mock
CMV-Cre
D)
Mock
Mock
CMV-Cre
CMV-Cre
Dicer
HSC70
Cre-GFP
Lin28a
(A177)
37kD
GFAP
Normalized Dicer Protein Level
Mock
CMV-Cre
Normalized Lin28a Protein Level
Mock
CMV-Cre
Normalized GFAP Protein Level
Mock
CMV-Cre
RFP
RFP
CMV-Cre
CMV-Cre
CMV-Cre
CMV-Cre
HSC70
Lin28B
(5422)
50kD
37kD
Normalized Lin28b Protein Level
Mock
CMV-Cre

Figure 2.5 Paralog specific effects on the miRNA profile in both GGAG as well as non GGAG miRNAs.

A) Log-log plot of average modified Top 100 normalized miRNA counts (excluding the Let-7s, mir-98, mir-107, and mir-9) in mock samples to fold change in normalized Lin28a fl/fl + CMV-Cre condition over Mock. GGAG pri-miRNA denotes collocated miRNAs. n=4 independently injected hippocampi from 4 different mice in each condition. **B)** Log-log plot of average miRNA counts normalized as in a) in mock samples to fold change in normalized Lin28b fl/fl + CMV-Cre condition over Mock. GGAG pri-miRNA denotes collocated miRNAs. n=4 independently injected hippocampi from 4 different mice in each condition. **C)** Log-log plot of average miRNA counts normalized as in a) in mock samples to fold change in normalized Lin28b fl/fl + CMV-Cre condition over normalized Lin28a fl/fl + CMV-Cre condition. GGAG pri-miRNA denotes collocated miRNAs. n=4 independently injected hippocampi from 4 different mice in each condition.

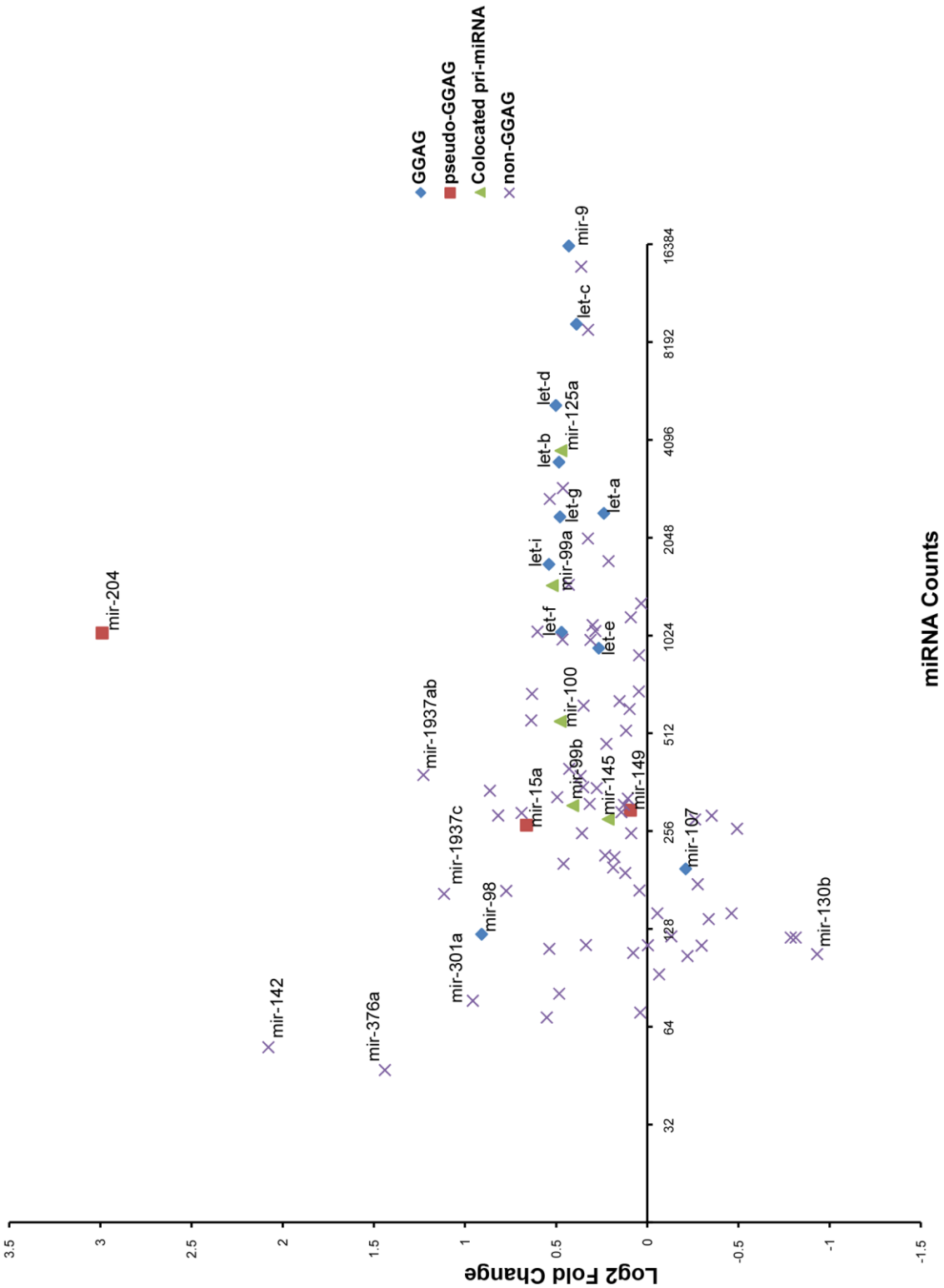
A)

Figure 2.5


GGAG
pseudo-GGAG
Colocated pri-miRNA
non-GGAG
miRNA Counts
Log2 Fold Change
1.5
1
0.5
0
-0.5
-1
16384
8192
4096
2048
1024
512
256
128
64
32
mir-9
let-c
let-d
mir-125a
let-b
let-a
let-g
let-i
mir-99a
mir-128
let-f
let-e
mir-100
mir-323
mir-204
mir-135a
mir-136
mir-15a
mir-145
mir-149
mir-99b
mir-107
mir-98
mir-21
mir-301a

B)

Figure 2.5


GGAG
pseudo-GGAG
Colocated pri-miRNA
non-GGAG
miR-204
mir-1937ab
mir-1937c
mir-301a
mir-98
mir-142
mir-376a
mir-15a
mir-100
mir-99b
mir-145
mir-149
let-f
let-e
let-i
mir-99a
let-g
let-b
let-d
mir-125a
let-a
let-c
mir-9
mir-107
mir-130b
Log2 Fold Change
miRNA Counts
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
-1
-1.5
32
64
128
256
512
1024
2048
4096
8192
16384

C)

Figure 2.5

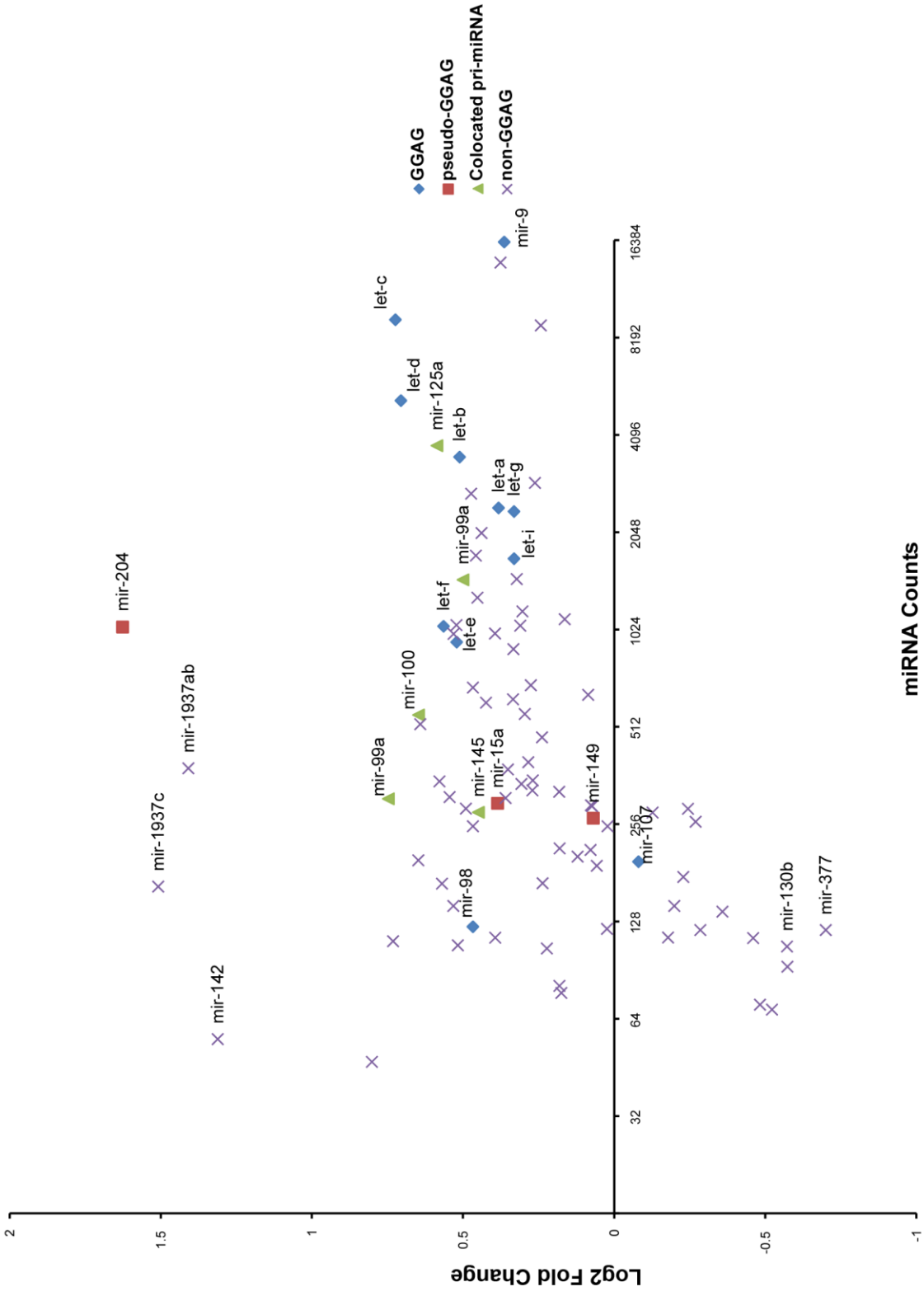

GGAG
pseudo-GGAG
Colocated pri-miRNA
non-GGAG
mir-9
let-c
let-d
mir-125a
let-b
let-a
let-g
let-i
mir-99a
let-f
let-e
mir-100
mir-99a
mir-145
mir-15a
mir-149
mir-107
mir-98
mir-204
mir-1937ab
mir-1937c
mir-142
mir-130b
mir-377
Log2 Fold Change
2
1.5
1
0.5
0
-0.5
-1
miRNA Counts
16384
8192
4096
2048
1024
512
256
128
64
32

Figure 2.6 Unsupervised hierarchical clustering of normalized miRNA profiles.

A) MiRNA profiles of 12 independent NanoString samples normalized to geometric mean of the top 100 detected miRNAs not previously known to be Lin28a regulated (excluding the Let-7s, mir-98, mir-107, and mir-9), then clustered agglomeratively and z-score transformed. GGAG, pseudo-GGAG, and collocated miRNAs indicated by colored markers (see extended experimental protocols appendix). **B)** Normalized only to housekeeping genes and ligation controls then clustered only for samples not miRNAs. Samples represent one Mock and two CMV-Cre injected hippocampi from 4 separate experiments, with each hippocampus also coming from separate mice. (see extended experimental protocols appendix) **C)** miRNA abundance correlations between independent samples are highest amongst those with the same treatment condition. Correlations of all samples with sample Mock 1 as the x variable (Top), Lin28a fl/fl + CMV-Cre 2 as the x variable (middle), and Lin28b fl/fl +CMV-Cre as the x variable (bottom).

Figure 2.6

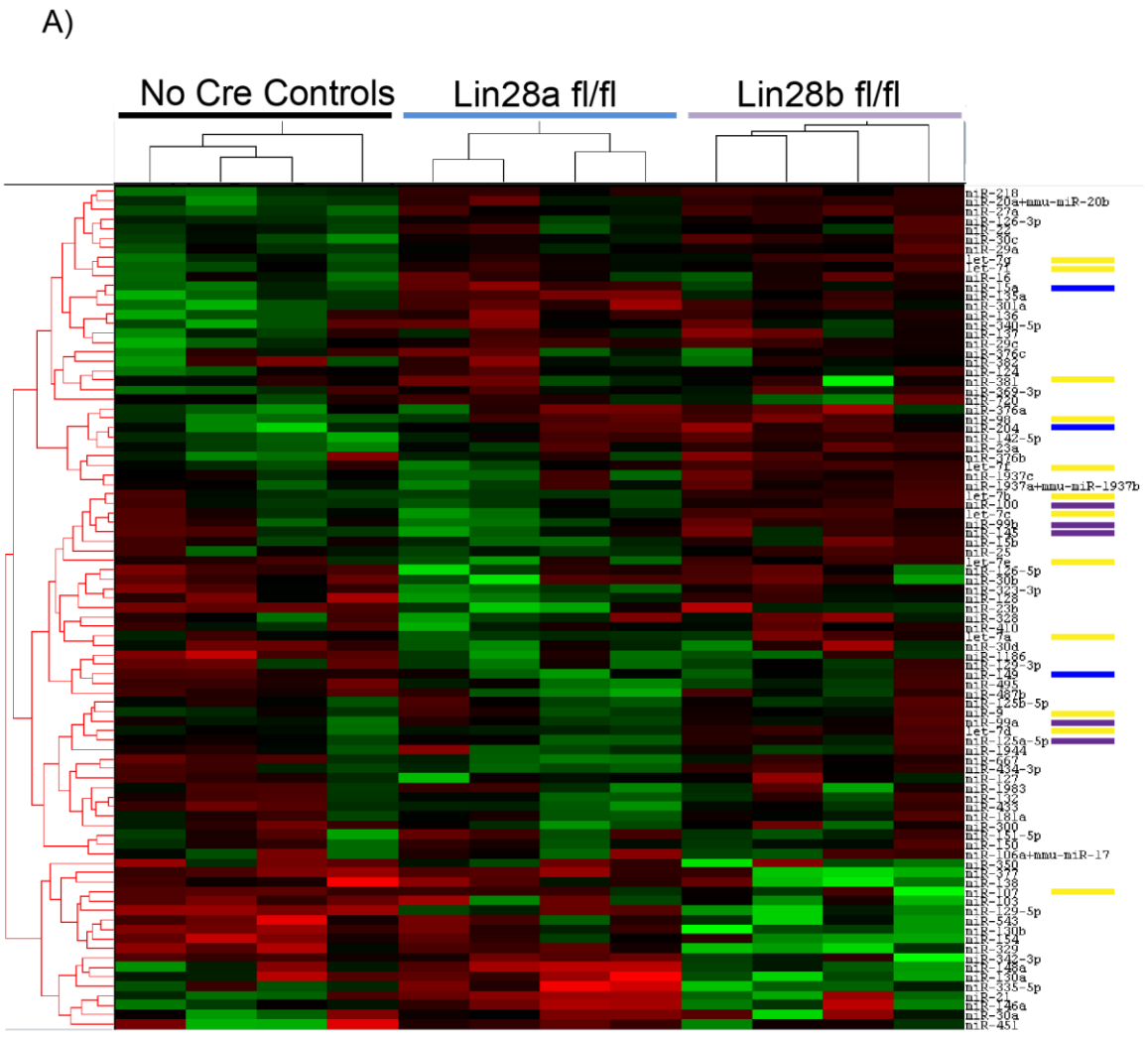
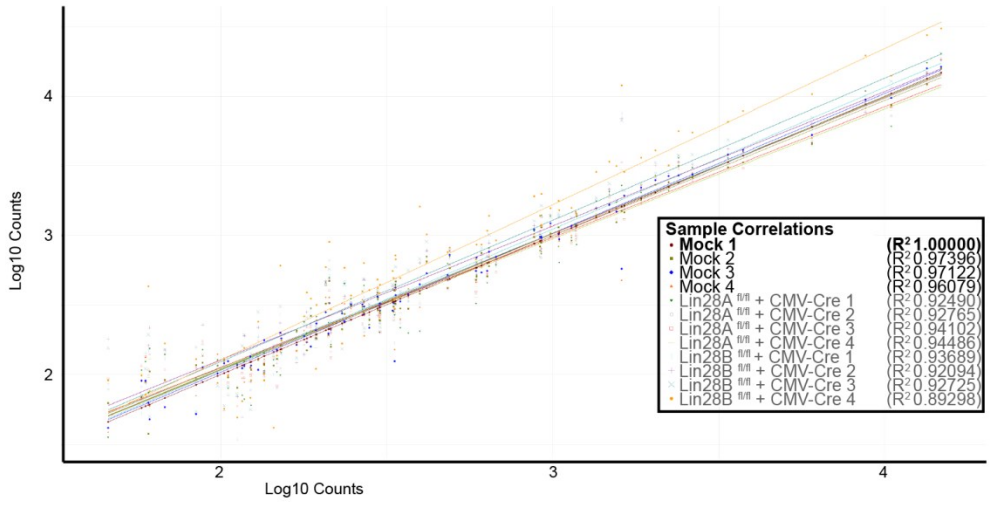
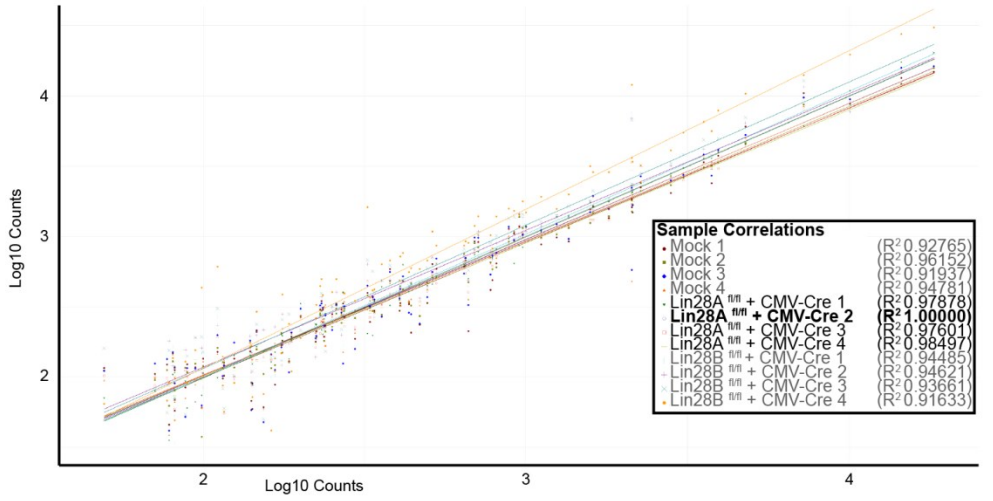
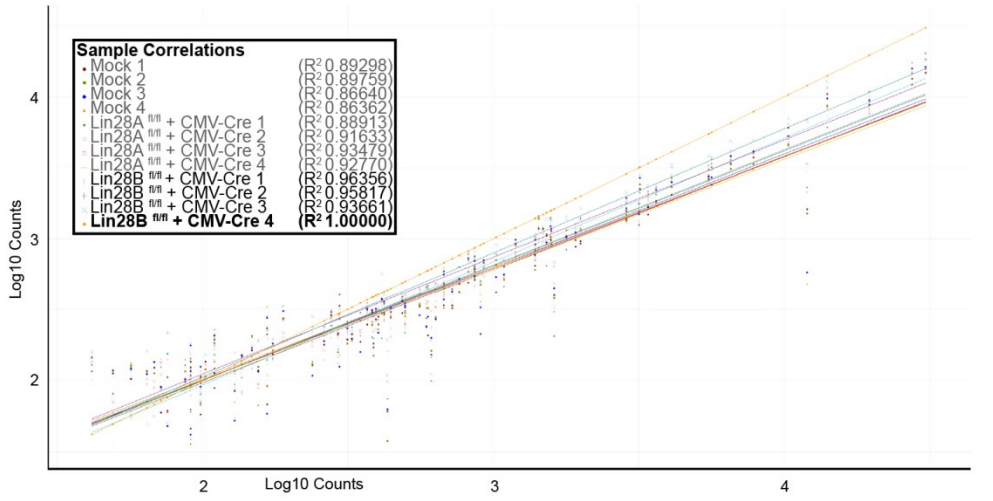

A)
No Cre Controls
Lin28a fl/fl
Lin28b fl/fl
B)
No Cre Controls
Lin28a fl/fl
Lin28b fl/fl
Let-7a
Let-7b
Let-7c
Let-7d
Let-7e
Let-7f
Let-7g
Let-7i
mir-100
mir-101b
mir-103
mir-106
mir-107
Z-Score
3.00
2.25
1.50
0.75
0
-0.75
-1.50
-2.25
-3.00
GGAG-miRNA
Pseudo-GGAG
Colocated pri-miRNA

Figure 2.6

C)


Sample Correlations
Mock 1 (R² 1.00000)
Mock 2 (R² 0.97396)
Mock 3 (R² 0.97122)
Mock 4 (R² 0.96079)
Lin28A fl/fl + CMV-Cre 1 (R² 0.92490)
Lin28A fl/fl + CMV-Cre 2 (R² 0.92765)
Lin28A fl/fl + CMV-Cre 3 (R² 0.94102)
Lin28A fl/fl + CMV-Cre 4 (R² 0.94486)
Lin28B fl/fl + CMV-Cre 1 (R² 0.93689)
Lin28B fl/fl + CMV-Cre 2 (R² 0.92094)
Lin28B fl/fl + CMV-Cre 3 (R² 0.92725)
Lin28B fl/fl + CMV-Cre 4 (R² 0.89298)
Log10 Counts
Log10 Counts
2
3
4


Sample Correlations
Mock 1 (R² 0.92765)
Mock 2 (R² 0.96152)
Mock 3 (R² 0.91937)
Mock 4 (R² 0.94781)
Lin28A fl/fl + CMV-Cre 1 (R² 0.97878)
Lin28A fl/fl + CMV-Cre 2 (R² 1.00000)
Lin28A fl/fl + CMV-Cre 3 (R² 0.97601)
Lin28A fl/fl + CMV-Cre 4 (R² 0.98497)
Lin28B fl/fl + CMV-Cre 1 (R² 0.94485)
Lin28B fl/fl + CMV-Cre 2 (R² 0.94621)
Lin28B fl/fl + CMV-Cre 3 (R² 0.93661)
Lin28B fl/fl + CMV-Cre 4 (R² 0.91633)
Log10 Counts
Log10 Counts
2
3
4


Sample Correlations
Mock 1 (R² 0.89298)
Mock 2 (R² 0.89759)
Mock 3 (R² 0.86640)
Mock 4 (R² 0.86362)
Lin28A fl/fl + CMV-Cre 1 (R² 0.88913)
Lin28A fl/fl + CMV-Cre 2 (R² 0.91633)
Lin28A fl/fl + CMV-Cre 3 (R² 0.93479)
Lin28A fl/fl + CMV-Cre 4 (R² 0.92770)
Lin28B fl/fl + CMV-Cre 1 (R² 0.96356)
Lin28B fl/fl + CMV-Cre 2 (R² 0.95817)
Lin28B fl/fl + CMV-Cre 3 (R² 0.93661)
Lin28B fl/fl + CMV-Cre 4 (R² 1.00000)
Log10 Counts
Log10 Counts
2
3
4

Discussion

The Lin28 paralogs are typically reported to undergo declining expression in late embryogenesis and to be undetectable or highly restricted in adult tissues except in progenitor cells (Yokoyama et al. 2008; D. H. Yang and Moss 2003; M. Yang et al. 2015). Unexpectedly, my data suggests that Lin28 paralogs might also be involved in early postnatal neural development. In addition to the significant differences in gross morphology of the hippocampus and size of the brain and body, postnatal ubiquitous deletion of Lin28a in the brain may lead to significant changes in cell fate determination similar to what has been found during early development and differentiation, neuronal cell death, or a combination of both (Balzer et al. 2010). Changes in cell fate could result from altered levels of Lin28 paralogs or from the effect that deficiency of the Lin28 paralogs has on levels of Dicer, and in the case of Lin28a KO, levels of Lin28b and possibly TRBP. Heterozygous and homozygous deletions of Dicer have been shown to have severe effects on brain development. Adult CaMKIIα-CreER^{t2} driven KO of Dicer leads to neuronal cell death in the hippocampus, and early CaMKIIα driven expression of constitutively active Cre leads to animal death around P21-24 (Vinnikov et al. 2014; Babiarz et al. 2011). Aberrant growth phenotypes might also result from de-repression of GGAG miRNAs, which one could speculate might lead to an abrupt early exit from pro-growth proliferative programs in the perinatal period. Interestingly, one of the upregulated pseudo-GGAG miRNAs, mir-15a, has been shown to inhibit BDNF mediated neuronal maturation (Gao et al. 2015).

The relatively modest Let-7 level changes at baseline in response to Lin28 KO are consistent with small changes seen in response to constitutive KO previously (Shinoda et

al. 2013; M. Yang et al. 2015). It is possible that over the course of prolonged KO homeostatic mechanisms downregulate let-7 levels that have increased immediately after loss of Lin28. It is also possible that let-7 levels are so high in neurons that there are significant hurdles to large increases. Indeed, in both Lin28a and Lin28b KO conditions, the magnitude of change in miRNA was negatively associated with an increase in baseline abundance. A small increase in a miRNA such as mir-9 represents the equivalent of an order of magnitude of change in a less abundant miRNA such as mir-98 in terms of absolute counts. In the case of mir-9 additionally, only 1 of 3 copies of the gene contains the GGAG motif. One of the copies not containing a GGAG motif is not expressed in brain, but the other one has the highest expression level of all three in brain (Shibata et al. 2011; Laneve et al. 2010).

It remains to be seen how these differences change in response to BDNF, it would be expected that a lack of Lin28 mediated downregulation would increase the differential between mock and Lin28 KO during a BDNF time course when compared to baseline, though the regulatory potential of the remaining Lin28 paralog could be significant if its response is unchanged on a KO background. Also of interest is the response of the newly posited Lin28 regulated miRNAs such as mir-204, mir-15a, mir-301, and the collocated GGAG-pri-miRNAs to BDNF stimulation. This could result in a more accurate predictive model of Lin28 mediated regulation in conjunction with previous data regarding pre-E-loop secondary structure and G-quartet binding in order to refine the GGAG definition bioinformatically (Elizabeth O'Day et al. 2015; Triboulet, Pirouz, and Gregory 2015).

Methods

Imaging: Acute slice were imaged with a 25x or a 40x 1.3 NA, EC Plan Neofluoar on a Yokogawa spinning disk (Cell Observer, Carl Zeiss) and tiled to capture the whole hippocampal cross-section in the Zen software. Images were analyzed with only the DAPI channel visible in order to blind the analysis to the expression of fluorescent virus. The distance measurement tool in the Zen software was used to determine the thickness or the respective cell body layers. Statistical significance was determined via Student's two tailed t-test with equal variance.

Immunoblotting: Whole hippocampi were dissected from P13 mouse pups and placed directly into Lysis Buffer (50 mM Hepes, 150 mM NaCl, 10% Glycerol, 1 mM EDTA, 1% Triton-X-100, 0.2% SDS) with fresh protease inhibitor (Roche) and phosphatase inhibitor cocktails (Sigma P004 and P5726). Lysates were then rotated at 4°C for 15 minutes, followed by high-speed centrifugation for 15 minutes at 12,000 X g also at 4°C. The supernatant was placed in a fresh tube and protein concentration was determined by Bicinchoninic acid (BCA) assay. Equal protein amounts were run on SDS-PAGE gels and electrotransferred to PVDF membrane. Membrane was blocked with 5% BSA in Tris-buffered saline tween 20 (TBST 0.1%) for 2 hrs at room termperature. Membranes were probed with primary antibodies: Lin28a (a177 Cell Signaling or LifeSpan-B11566), Lin28B (Cell Signalling 5422), Dicer (Sigma SAB4200087), HSC70 (Santa Cruz sc-7298), TRBP (Abcam ab72110), GFAP (neuromab n206A8), GFP (NeuroMab N86/8) or btubIII (U of Iowa DSHB, E-7). Primary was washed off 3x for 15 minutes in TBST, then incubated for 1 hour in 1:10,000 secondary antibody in 5% BSA TBST. Secondary washed 3x 10 minutes in TBST before 2 minute incubation in Pierce ECL and exposure

to western blotting film and subsequent developing.

miRNA RT qPCR

Total RNA from one half of each P13 hippocampal lysate was isolated in Tri-Reagent (Molecular Research Center, Inc.) according to the manufacturer's protocol. RNA pellets were air-dried and resuspended in 25 uL nuclease-free water. RNA concentration and quality were assayed by optical density (OD) at 260/280/230nm on a NanoDrop.

20ng of total isolated RNA was used for reverse transcription using candidate miRNA specific TaqMan (Applied Biosystems) primers in 15µl reactions according to manufacturer's instructions: 4^{o}C for 5 min, 16^{o}C for 30 min, 42^{o}C for 30 min, 85^{o}C for 5 min. CDNAs were then assayed via TaqMan miRNA assays for Let-7a (000377), Let-7c (000379), and miR-132 (000457). The amount of u6 (002282) in each sample was used as a control to normalize all miRNA species. RT qPCR was performed on a Stratagene Mx3000P machine and accompanying software. Quantification was carried out using the standard-curve method.

Genotyping PCR Genomic DNA was extracted from either mouse tails (genotyping PCR) or remaining hippocampal lysate and nuclear pellet (recombination PCR) using phenolchloroform extraction. For Lin28a fl/fl recombination the forward primer was 5'-TCC AAC CAG CAG TTT GCAG-3' and the Reverse Primer was 5'-AAT ACA ACC TTA GTT TCT-3'. For Lin28B genotyping the forward primer was 5'-AAC GCA CAT TGC AAA TAC CC -3' and the reverse primer was 5'-AAC TCG AGT TAT GTT GTA CC-3'. See extended methods and protocols for cycling program and pipetting information.

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Chapter III: Characterization of Postnatal Neuron Specific Knock Out of Lin28a

Background

Ongoing work in the Meffert lab has uncovered a growing body of evidence that Lin28a plays a crucial role in neuronal growth and plasticity. Lin28a confers specificity to and is necessary for the regulation of gene target specificity in protein translation in response to BDNF. Furthermore, Lin28a also is necessary and sufficient for the neuronal growth effects of longer BDNF exposure. Much of this work has been done in primary hippocampal neuronal culture, and raises the interesting question of the relative prevalence of Lin28a in the diverse cellular population of the hippocampus, and the specific importance of neuronal Lin28a in that context.

Previous results from ubiquitous postnatal deletion of Lin28a in brain using P0 injected AAV CMV Cre-GFP discussed in Chapter 2 suggested a possible trophic or developmental requirement for Lin28a. Lower survival (1 of 7 injected survival to P21 compared with 9 of 11 Mocks, 7 of 15 and 6 of 7 respectively for P17) and strongly induced levels of GFAP raised the possibility of an apoptotic response to loss of Lin28a. In order to investigate the validity of this limited observation, we chose to determine whether an apoptotic response would be observed in the context of excitatory neuron-specific postnatal reduction of Lin28a levels. We investigated this question using a mouse line expressing CreERT2 under the control of the forebrain neuron-specific promoter, CaMKIIα (CaMKIICreER^{T2}), which was crossed to the Lin28a$^{fl/fl}$ line from the Daley lab(Shinoda et al. 2013). to produce a new line (CaMKII/Lin28af/f) allowing

inducible recombination at the Lin28a locus as described in Figure 3.1b. (from the Guenther Schuetz lab diagrammed in Figure 3.1a (Erdmann, Schütz, and Berger 2007).

Results

Induced Postnatal Lin28a deficiency in Hippocampal Neurons does not lead to Significant Increases in Apoptosis.

In order to determine whether loss of neuronal Lin28a leads to an apoptotic response by the time we detected significant differences in brain size and hippocampal gross morphology in ubiquitous Lin28a deficiency, we harvested our experimental litters on postnatal day 13 (P13). 4-hydroxytamoxifen (OHT) 75mgs/kg to induce Cre activity was delivered by daily IP injection beginning at day 8. Recombination at the genomic locus was readily detectable after 5 days of treatment with OHT, yet whole hippocampal lysates did not show a reduction in Lin28a or Dicer levels when compared to littermate control animals (Fig3.1c). RNA extracted from whole hippocampal lysates showed mild increase in Let-7c trending toward significance and significant changes in mir-132 (Fig3.1c). This led us to use a more neuron specific assay of Lin28a loss, in case neuronal Lin28a may represent a smaller fraction of the hippocampal pool at baseline. It is also possible that glial upregulation of Lin28a in response to stress from neuronal loss of Lin28a could obscure the change.

We fixed and cryosectioned matched hippocampi from experimental and control animals, and assayed Lin28a immunofluorescence in the CA2 neuronal cell body layer (3.1d). Hippocampi were matched dorso-ventrally prior to sectioning and the same subsection of neurons was assayed because neuronal gene expression can vary widely both by hippocampal area as well as along the dorso-ventral axis (Cembrowski et al.

2016). In contrast to whole hippocampal lysates, Lin28a signal detected in neurons by immunohistochemical staining was significantly reduced (down to 55% of mock injected Lin28a levels P=0.0044, n=4 mock, 6 OHT sections) in volumes masked by detection of neuronal marker NeuN (Fig. 3.1e). Control staining in the molecular layer, masked by exclusion from NeuN signal in order to eliminate any interneuron or nonspecific staining, was slightly but not significantly increased (n=3 mock, 3 OHT).

Apoptosis was assayed via TUNEL staining in the same fixed, cryosectioned hippocampi. Nuclei were visualized via DAPI, and TUNEL positive nuclei per imaged hippocampal section counted. TUNEL signal was not significantly increased in OHT treated animals in comparison to vehicle treated littermates when normalized to DAPI counted nuclei, and neither when masked in NeuN nor overall.

Discussion

In this study a small but not statistically significant increase in apoptotic nuclei was detected in response to a decrease in neuronal Lin28a, which could possibly reach significance with increased n, but physiologically relevant increases in apoptosis are usually much higher. These results could suggest that apoptosis induced by neuronal loss of Lin28a might not be the cause of the phenotypes seen in response to ubiquitous Lin28a deficiency by viral Cre injection in Chapter 2; however, there are several caveats to this interpretation: 1) Lin28a deficiency was assessed using IHC which is a semi-quantitative approach, 2) Lin28a-deficiency assessed in neuronal cell bodies by IHC was significant (45% +/- 8% reduction), but it is unknown if this represents a biologically significant reduction in Lin28a function, 3) There was no reduction of Lin28a staining observed in the molecular layer where there are still neuronal processes and synapses along with glia 4) No timeline was conducted, so it is unclear for how long the neurons were deficient in Lin28a and whether a longer period of deficiency might have produced a different physiological outcome, and 5) Lin28a deficiency was assessed only in basal conditions and it is not yet clear whether a significant change would also be observed under excitation which induces Lin28a. A change in Lin28a levels was not detected by total hippocampal lysates, which might suggest that at baseline, either Lin28a not in neuronal cell somas (ie in processes) or non-neuronal Lin28a may make up a majority of the hippocampal pool, or simply that there is a technical difference in the sensitivity or specificity of the two approaches (IHC compared to immunoblotting).

However, this study is far from conclusive. In the previous chapter, CMV-Cre was injected at P0 and expected to be expressing and recombining at an earlier time point

than the first OHT injection in the current study. It is possible that recombination even one or two days earlier in development could significantly alter the effects seen at P13. Similarly, it is possible that prolonged time of Lin28a loss through later tissue harvest would lead to a pronounced apoptotic phenotype. Further imaging, and costaining with GFAP and miRNA biogenesis components like Dicer could reveal whether neuronal loss of Lin28a has non-apoptotic non-cell-autonomous effects. Furthermore, microdissection of neuronal cell body layers of the hippocampus could reveal more granularly the effect of CaMKII-CreERT2 mediated loss of Lin28a on the miRNA biogenesis machinery and miRNA levels in neurons compared with the remainder of the hippocampus.

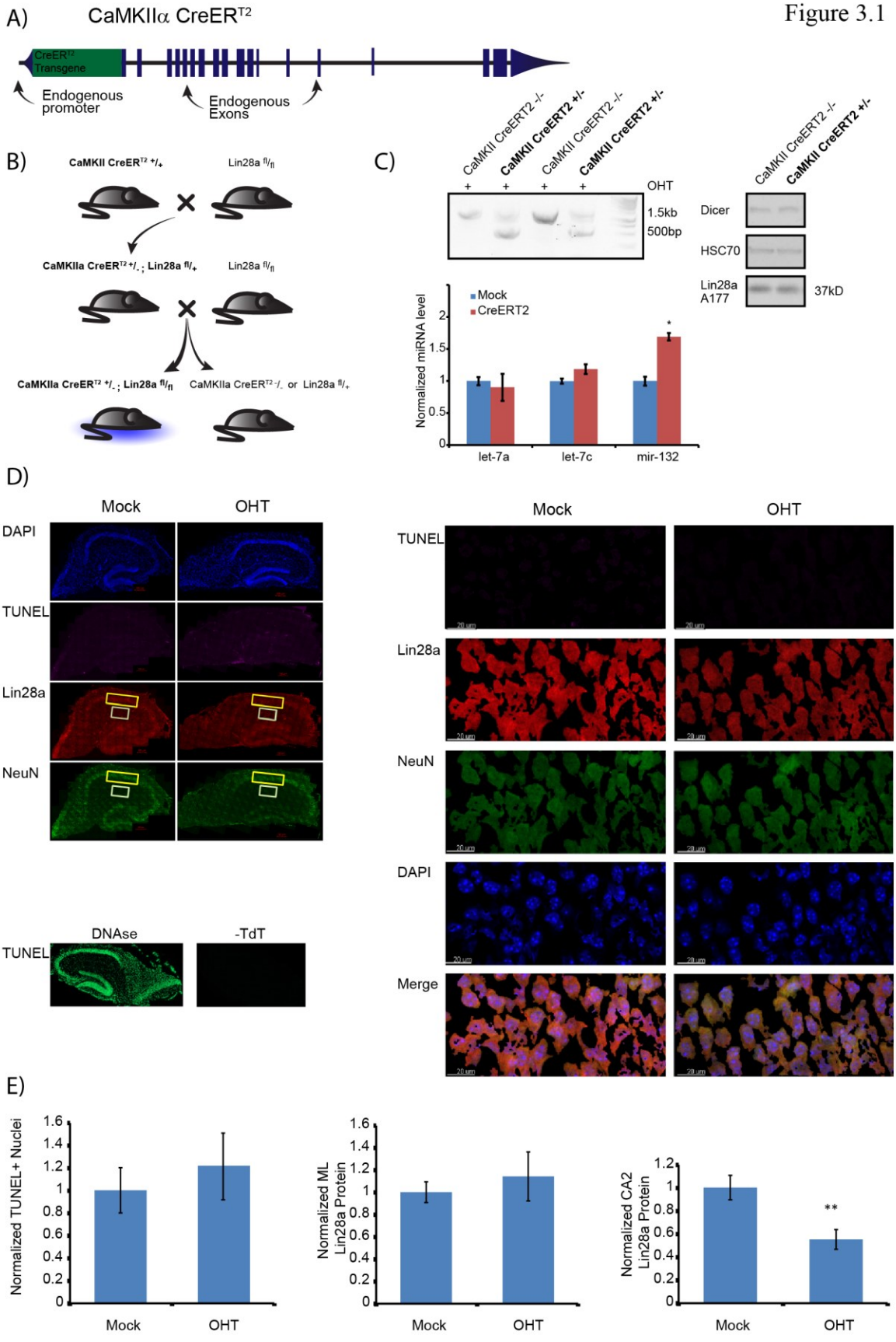
Figures and Legends

Figure 3.1 Induced Postnatal Lin28a deficiency in Hippocampal Neurons does not lead to Significant Increases in Apoptosis.

A) Diagram of genomic locus of CaMKII-Cre transgene. Green denotes exogenous transgene, endogenous exons denotes CaMKIIα **B)** Mating approach for mouse model. Litters were either all injected with OHT and experimental and control groups determined by transgene genotype (panel C)), or genotyped prior to injection (panels d) and e)). In the latter case only CreERT2 transgene positive Lin28a floxed mice (blue in panel b)) were selected and injected with either vehicle (sunflower seed oil) or 4-OHT. **C)** Whole hippocampal outcomes from neuron specific recombination. Representative recombination PCR for CreERT2 positive hippocampi (top left). Assay is the same as recombination PCR assay shown in Figure 2.2b. All mice received OHT. Immunoblot for Lin28a and Dicer protein levels (top right) shows no significant differences in whole hippocampal lysates from P13 mice receiving daily IP injetions of OHT from P8 to P12 and miRNA qPCR results for candidate miRNAs normalized to U6 snRNA (bottom left, n=4 and 2 hippocampi respectively). **D)** IHC and TUNEL staining of hippocampal sections. Representative images of hippocampal sections from P13 pups mock or OHT injected (top left). Yellow rectangle represents CA2 region selected for ROI analysis. Green rectangle represents molecular layer region selected for control ROI. TUNEL assay controls with either DNAse added or no labeling enzyme (bottom left). Representative images of ROIs masked by NeuN expression for Lin28a quantitation (right). **E)** Quantification of imaging studies represented in panel d). Analysis was done

blinded to the treatment status of the slices. Statistical significance determined via two tailed Student's t-test * $p < 0.05$ ** $p < 0.01$. Error bars represent SEM.

Figure 3.1


A)
CaMKIIα CreER^{T2}
CreER^{T2} Transgene
Endogenous promoter
Endogenous Exons
B)
CaMKII CreER^{T2} +/+
Lin28a $^{fl/fl}$
CaMKIIa CreER^{T2} +/-; Lin28a $^{fl/+}$
Lin28a $^{fl/fl}$
CaMKIIa CreER^{T2} +/-; Lin28a $^{fl/fl}$
CaMKIIa CreER^{T2} -/- or Lin28a $^{fl/+}$
C)
CaMKII CreERT2 -/-
CaMKII CreERT2 +/-
CaMKII CreERT2 -/-
CaMKII CreERT2 +/-
OHT
1.5kb
500bp
Dicer
HSC70
Lin28a A177
37kD
Mock
CreERT2
Normalized miRNA level
let-7a
let-7c
mir-132
D)
Mock
OHT
DAPI
TUNEL
Lin28a
NeuN
DNAse
-TdT
Merge
E)
Normalized TUNEL+ Nuclei
Normalized ML Lin28a Protein
Normalized CA2 Lin28a Protein
Mock
OHT

Methods

***Tamoxifen treatment*:** For imaging studies, P8 CaMKII-CreERT2 +/, Lin28a fl/fl pups were injected with 75mgs/kg 4-OHT in 100uL sunflower seed oil daily for 5 days. Controls were injected with 100uL sunflower seed oil for the same period. A total of 6 mice from 2 litters were used, 2 treated and 1 control from each litter. For miRNA qRT PCR, Western blotting and genomic recombination PCR, P8 Lin28a fl/fl; CaMKII-CreERT2 +, and P8 Lin28a fl/fl; CaMKII-CreERT2 – animals were both injected with 75mgs/kg 4-OHT in 100uL sunflower seed oil daily for 5 days. 6 animals from 1 litter were used, 2 positive for the CreERT2 transgene and two negative. Both of these approaches have previously been used equivalently with this CaMKII-driven CreERT2 mouse line (Lagerlöf et al. 2016).

Sectioning and IHC: On P13, pups were sacrificed and hippocampi were fixed in 4% paraformaldehyde overnight at 4^{o}C. Hippocampi were then cryoprotected in 30% sucrose overnight at 4^{o}C. Treatment and control hippocampi were embedded in OCT side by side in order to match sections dorsoventrally. 16uM sections were collected on slides and washed 3 times with PBS (pH 7.4). Sections were then blocked and permeabilized for 2 hours in a humidified chamber at room temperature in 10% NGS, 0.3% Triton, PBS (10% NGST). Sections were then incubated in primary antibodies diluted in 1% NGST overnight at room temperature (Cell Signaling A177 Rabbit Lin28a 1:100, Millipore Mouse NeuN 1:200). Slides were washed 3 times in PBST for 10 minutes. Slides were then placed in TUNEL equilibration buffer for 10 minutes (TUNEL Dead End Fluorometric System from Promega). Slides were then treated with equilibration buffer with 8% TUNEL kit nucleotide mix and 2% TdT enzyme along with 1:1000 dilution of

fluorescent secondary antibodies (Alexa 568 anti-rabbit Alexa 633 anti-mouse) for 1 hour in dark humid chamber at room temperature. Positive TUNEL controls were pre-treated with DNAse for 10 minutes and processed separately to avoid residual DNase activity as per manufacturer instructions. Negative controls were treated identically in the absence of TdT enzyme. Slides were placed in 2x SSC for 15 minutes to stop the TUNEL reaction, then washed 3 times with PBS at room temperature. 2 drops of DAPI were added to the top of each slide and incubated in the dark for 5 minutes (ThermoFisher NucBlue Fixed Cell imaging kit) Slides were covered in DAKO mounting media and coverslips secured with clear nail polish and stored protected from light at 4^{o}C.

Imaging: Stained sections were imaged with a 40x 1.3 NA, EC Plan Neofluoar on a Yokogawa spinning disk (Cell Observer, Carl Zeiss) and tiled to capture the whole hippocampal cross-section in the Zen software. TUNEL positive nuclei were analyzed via Particle Analyzer in Image J masked for DAPI positive nuclei as determined by Particle Analyzer. Lin28a fluorescence in NeuN costained neurons was quantified in Imaris (BitPlane). Briefly, an ROI was selected in CA2 and NeuN signal was used to make a mask using the Surface tool. This mask was used to analyze Lin28a expression within NeuN marked neurons using average signal intensity within the mask. For molecular layer controls, a region between CA2 and the dentate gyrus with low NeuN signal was chosen, and Lin28a signal was assayed in a reverse NeuN mask to exclude any interneurons or blood vessel related nonspecific signal. Each individual hippocampal section where an ROI was imaged was treated as a statistical n, with sections treated with OHT representing 4 mice from 2 litters and their paired mock treated sections

representing 2 mice from those two litters. Statistical significance was determined via Student's two tailed t-test with equal variance.

Immunoblotting: Whole hippocampi were dissected from P13 mouse pups and placed directly into Lysis Buffer (50 mM Hepes, 150 mM NaCl, 10% Glycerol, 1 mM EDTA, 1% Triton-X-100, 0.2% SDS) with fresh protease inhibitor (Roche) and phosphatase inhibitor cocktails (Sigma P004 and P5726). Lysates were then rotated at 4°C for 15 minutes, followed by high-speed centrifugation for 15 minutes at 12,000 X g also at 4°C. The supernatant was placed in a fresh tube and protein concentration was determined by Bicinchoninic acid (BCA) assay. Equal protein amounts were run on SDS-PAGE gels and electrotransferred to PVDF membrane. Membrane was blocked with 5% BSA in Tris-buffered saline tween 20 (TBST 0.1%) for 2 hrs at room termperature. Membranes were probed with primary antibodies: Lin28a (a177 Cell Signaling), Dicer (Sigma SAB4200087), or HSC70 (Santa Cruz sc-7298).

miRNA RT qPCR

Total RNA from one half of each P13 hippocampal lysate was isolated in Tri-Reagent (Molecular Research Center, Inc.) according to the manufacturer's protocol. RNA pellets were air-dried and resuspended in 25 uL nuclease-free water. RNA concentration and quality were assayed by optical density (OD) at 260/280/230nm on a NanoDrop.

20ng of total isolated RNA was used for reverse transcription using candidate miRNA specific TaqMan (Applied Biosystems) primers in 15µl reactions according to manufacturer's instructions: 4^{o}C for 5 min, 16^{o}C for 30 min, 42^{o}C for 30 min, 85^{o}C for 5 min. CDNAs were then assayed via TaqMan miRNA assays for Let-7a (000377), Let-7c (000379), and miR-132 (000457). The amount of u6 (002282) in each sample was used

as a control to normalize all miRNA species. RT qPCR was performed on a Stratagene Mx3000P machine and accompanying software. Quantification was carried out using the standard-curve method.

Genotyping PCR Genomic DNA was extracted from either mouse tails (genotyping PCR) or remaining hippocampal lysate and nuclear pellet (recombination PCR) using phenolchloroform extraction. For Lin28a fl/fl recombination the forward primer was 5'-TCC AAC CAG CAG TTT GCAG-3' and the Reverse Primer was 5'-AAT ACA ACC TTA GTT TCT-3'. For CaMKII-CreERT2 genotyping the forward primer was 5'-GAC AGG CAG GCC TTC TCT GAA-3' and the reverse primer was 5'-CTT CTC CAC ACC AGC TGT GGA-3'. See extended methods and protocols for cycling program and pipetting information.

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Chapter IV: Conclusions and Perspectives

Coordinated modulation of synaptic strength is required for effective learning and memory. The rapid, specific, changes in gene expression necessary for lasting changes in synaptic strength make accurate post-transcriptional control of protein synthesis important. Regulation of gene expression by miRNAs has emerged as a major pathway of post-transcriptional control in the brain. The combinatorial power of miRNAs being able to target ensembles of proteins allows for signal amplification via regulation of only a small number of miRNA species. Our lab has shown that the regulation of Let-7 miRNAs by Lin28a is central to the specificity of neuronal gene expression in response to BDNF. Lin28a had been thought to no longer be expressed later in development and in differentiated tissues. The downregulation of Let-7s is necessary for the increase in pro-growth synaptic proteins within minutes of BDNF, as well as for the long term growth of the dendritic arbor of neurons. The BDNF pathway is vital for the growth and survival of neurons, and required for some forms synaptic plasticity. In recent years, dysregulation of the BDNF pathway has been discovered in neurodevelopmental and psychiatric disease, and enhancement of BDNF signaling has been studied as a possible therapeutic target for neurodegenerative disease.

Lin28 was originally discovered as a critical regulator of developmental timing, and the absence of Lin28a during embryogenesis has been shown to be lethal in both vertebrates and invertebrates (Shinoda et al. 2013; Moss, Lee, and Ambros 1997). In addition to Lin28a, vertebrates have a second Lin28 paralog, Lin28b. Lin28b is similar to Lin28a in sequence and mode of action, with subtle differences. Lin28b has been shown to harbor nuclear localization sequences, and thus be localized separately from Lin28a

which is primarily cytosolic. Additionally, Lin28b regulates a different step of Let-7 biogenesis and was not shown to be post-transcriptionally induced by BDNF. In my thesis work, I aimed to characterize more fully the differences between Lin28a and Lin28b in their effects on the miRNA profile in the brain. To avoid the severe developmental effects associated with prenatal loss of Lin28, I decided to investigate the effects of Lin28 using a conditional postnatal deletion model.

Surprisingly, the experimental setup generating loss of either Lin28a or Lin28b in brain postnatally resulted in growth defects, suggesting postnatal preservation of functional importance for the paralogs. An important caveat of these experiments is that they have yet to be rigorously controlled for potential deleterious effects of viral overexpression of Cre, which could be independent of deficiency of Lin28 paralogs. A dose-titration of Cre and examination of sufficient numbers of transgenic and wildtype mice at the lowest effective Cre doses would be required.

Conditional knock out of Lin28a, but not Lin28b, possibly recapitulates a prenatal developmental effect on neurogliogenesis. Treatment of Lin28a$^{fl/fl}$ mice with CMV-Cre led to large increases in GFAP. In previous studies, overexpression of Lin28a had blocked increases in GFAP in in a model of glial differentiation and promoted neurogenesis in mouse embryos (Balzer et al. 2010; M. Yang et al. 2015). However, these increases have yet to be mechanistically linked to Lin28a loss. Additionally, the miRNA biogenesis protein Dicer is reduced in postnatal deficiency of Lin28a or Lin28b, while changes in Dicer's partner protein TRBP don't yet reach statistical significance. This reduction in miRNA biogenesis machinery is reflected in the miRNA profiles of whole hippocampus, as total detected miRNA counts were reduced in response to loss of

either Lin28 paralog. Interestingly, though requiring further experiments for confirmation, miRNA profiling revealed a possible addition to the suite of miRNAs regulated by the Lin28 paralogs via a GGAG motif. Mir-204, a neuron enriched highly expressed brain miRNA was detected at much higher levels in both Lin28a and Lin28b conditional knockouts.

The second part of my doctoral work investigated whether the growth defects seen in response to Lin28a loss could be due to apoptosis induced by loss of Lin28a in neurons. The severe trophic effects of loss of BDNF signaling and the importance of Lin28a in the BDNF pathway made this a hypothesis an attractive candidate to explain the differences in growth and the possible induction of glia. When assayed at the same developmental age as the mice with ubiquitously induced conditional deficiency in Lin28a, however, there was no detected increase in apoptosis in the hippocampus. Future work could investigate whether prolonged deletion of Lin28a either through earlier induction of recombination or later harvest of tissue does result in increases in apoptosis because only a limited set of conditions was tested.

Clearly, the functional relevance of the Lin28 paralogs postnatally in the brain represents a promising target for the investigation of a wide variety of pathologies. Testing the roles of the loss of the Lin28 paralogs in adult mice to see if there are effects on neurogliogenesis, as well as memory and learning behaviors will be an important endeavor. Further examination of the cell type specific phenotypes of Lin28 loss, could extend the neurological importance of Lin28 into glial cell types which represent a majority of the cells in the brain. Finally, the well-studied roles of Lin28 in both vertebrate and invertebrate development make the Lin28 / Let-7 pathway an interesting

prospect for study of neurological diseases, particularly those thought to have a developmental component.

Extended Experimental Protocols

PCR Genotyping:

Lin28 Paralog fl/fl Recombination PCR

Component	Amount (uL)
Buffer:	2.5
dNTPs:	1
Taq:	0.25
Template (~1ug/ml):	3
Primers (4uM):	5
Water	13.25

Primer	Sequence 5' --> 3'	Primer Type
Lin28aF	TCC AAC CAG CAG TTT GCAG	Forward
Lin28aR2	AAT ACA ACC TTA GTT TCT	Reverse

Primer	Sequence 5' --> 3'	Primer Type
Lin28bF	AAC GCA CAT TGC AAA TAC CC	Forward
Lin28bR3	AAC TCG AGT TAT GTT GTA CC	Reverse

PCR Thermocycler Protocol:

95 5 min

95 0:30 sec

56 0:30 sec

72 2 min

x 35

72 10 min

8 hold

Band Expected:

Lin28A:

Unrecombined: ~1250bp (Sequence below + ~250bp of two LoxP sites)

TCCAACCAGCAGTTTGCAGGTTCGAGCTTGCGATTCAGCGGGCACACCTTAGGGTGCAGGGAGCCCAATGT

CTAAATAGAAGGGAGGTGGGAAAGTGTCCCACGGACTTGTCCGCGGGACCCCCAAGTCCCTATGGCCTCCT

GCTTCTGGGGGCCATCTCTGGACTAGGGAACCCTGGTGTTCCAGTGGCTTGTACACCGAGCCCGTGGGGGC

GGAGGGAGAGAAGAGGAGGGGCCTGTCCCTCCGCGTGTGCACGGGCAACTTGTTGTGGGAAGACAAGCAAA

ACTTTTGGGGCGTCAGCATCCTCTGTGTAAGCTTGGGGAGGCAGCCAGGACAGGTTTCTTCTTACCAGCTG

CTTGGTAGCTGCCTCCTCCTGGGTGTCTATCCGCGGGCTGACCACCGAGATCTCCCAGACGGGTCCCTGAG

AGGGCCTGCGATCCCTTGGCCCCCAGCTGATCACCTGGCCCTCCCTATCTCCAGGTGGCTGCGCCAAGGCA

GCGGAGAAGGCGCCAGAGGAGGCGCCGCCTGACGCGGCCCGAGCGGCAGACGAGCCGCAGCTGCTGCACGG

GGCCGGCATCTGTAAGTGGTTCAACGTGCGCATGGGGTTCGGCTTCCTGTCTATGACCGCCCGCGCTGGGG

TCGCGCTCGACCCCCCGGTGGACGTCTTTGTGCACCAGGTGAGACTCATTCTGGAACTTTGCTAGGGGAAA

AAAAAGAAAAAGGCTGGGAGTTCCGAGGTACGGCCTTGGCTGGTGGCCCCTCCTGTCGGATTGGAGGAGAT

AAATACCCCTCATTGTGCGTCCCCATATTTTTCGGGACACCCCAGTTTTGAGTTTGTAGTAACTGCAGTTG

TGCGAGGAGGCACTGCTGACTACCAGCACCCCCTCCCCGCCCCCTTCCGGGAACCCCTCCGGCTTACCACG

TGTCTATTACCTCTTTCCCCTGGGAAGGGAGCAGGGGGCAGGGAGGTGACCCCATGT**GGCAGAAACTAAGG**

TTGTATT

Recombined: ~400bp

Lin28B:

Unrecombined: ~1150bp (Sequence below + ~250bp of two LoxP sites)

AACGCACATTGCAAATACCCCGGGATTCCCGTCTGCCTCATGTGCATTAACCAGTAGCCAGCCATCCTTAG
GATTTCTCTTTTTTTCTTTCTCCTTGCTAGGAAAAGCAATCTCGCTGAAAAATTACTCTTTGTAAATTGGA
ATAGTATTAGATCAGCAGCCTGTGAGTGAAGTGTCAGAAGCTGTAACCGTAGAATGTTGCCGAGAAAGGAG
CCAGATGAAAAAGTCTGCGGCTGTACGGAAACGTGGAAGCTGCTGTGAGCAGTGCAGCAATGTGGCTTCCC
CTCCCTGGCTTTGCTTTTAACCCTTTTTGTTTCAATTTTTTTTTTTAAGAAGGAAAATGTTTGCCAACCTTT
TGTTTTTAATCTTCATGTCTTCTTTTCCCAAGCAAAACTTTTTGTTTAGATGAGCACTGTATAGAAATGCA
GGCTAACTGACTTTTGTCCTCTTCCGTGTCAG**GCGGGGCAAGCAAAGGTGAAGAGCCAGAAAAACTGCCCG**
GGCTGGCAGAGGACGAACCCCAGGTTCTGCATGGCACTGGCCACTGTAAATGGTTCAACGTGCGCATGGGA
TTCGGATTCATCTCCATGATAAGTCGAGAGGGAAATCCCTTGGATATTCCAGTGGATGTATTTGTACACCA
AGTAAGCCAGACTTTTTAATCCCTTTTTTACCGGATTCCATGCTTGGGAGTTGGTGTGTAGTTATGTTAAT
ATATATAGAAAAATGTGCTTTTAAGTGTATTGTAATACGGAAATATTCGATAAAAATCAGGTGATTGTCCA
GTTTTCAGATGTGTCCCTGCTGGGGTATATTTTTATAAATAGAAAATGAAGGCTGGTACTTCACTGACT**GG**
TACAACATAACTCGAGTT

Recombined: ~350bp

Immunoblot:

Whole hippocampi were dissected from P13 mouse pups and placed directly into Lysis Buffer (50 mM Hepes, 150 mM NaCl, 10% Glycerol, 1 mM EDTA, 1% Triton-X-100, 0.2% SDS) with fresh protease inhibitor (Roche) and phosphatase inhibitor cocktails (Sigma P004 and P5726). Lysates were then rotated at 4°C for 15 minutes, followed by high-speed centrifugation for 15 minutes at 12,000 X g also at 4°C. The supernatant was placed in a fresh tube and protein concentration was determined by Bicinchoninic acid

(BCA) assay. Equal protein (~65ug/lane if possible from yield) amounts were run on SDS-PAGE gels and electrotransferred to PVDF membrane. Membrane was blocked with 5% BSA in Tris-buffered saline tween 20 (TBST 0.1%) for 2 hrs at room termperature. Membranes were probed with primary antibodies: Lin28a (a177 Cell Signaling), Dicer (Sigma SAB4200087), or HSC70 (Santa Cruz sc-7298).

P0 Injections in Lateral Ventricle:

Note: For Experiments where dense, comprehensive injection with virus is required, use only pups born within the last few hours, for experiments expressing fluorophore for imaging where relatively sparser labeling is acceptable, pups may be up to one day old.

AAV should be aliquoted in ~15 uL aliquots upon first opening to avoid freeze thaw cycles. Fill 50ml conical halfway with ice, insert viral aliquots, and top with ice for transport to animal facility.

Fast Green Dye can be diluted 1:10 in viral PBS and still be visible upon injection for quality control of viral spread.

Glass micropipettes should be kept sterile after pulling, and micro-injector syringe should we cleaned with ethanol and dried after use.

1. Anesthetize pup prior to injection by placing on ice for ~90-120s, until no longer moving.
2. Place 1-2ul of AAV-fast green mix (depending on number of co-injected viruses and titer) on parafilm and suction into micropipette.

3. Inject, while holding micropipette perpendicular to skull surface, at a point about 2/3 the way from eye spot to lambda. Repeat for opposite hemisphere.

4. When finished, warm pups in hand or on warming pad until pink and moving again. Place in previous nesting and dab with small amount of dam or foster dam's urine.

NanoString Profiling Analysis

Raw data normalization:

1. Load raw data files into nSolver Software
2. Load Samples to normalize as New Experiment
3. Annotate samples as desired for later ease of ratio comparison
4. For background subtraction, select user definable value and subtract the value for the highest negative control.
5. For normalization, select Codeset content, and then Top 100. After selection of Top 100, remove any a-priori expected targets and replace with next highest probes. (OR Normalize to housekeeping genes and assay-internal ligation controls)
6. Select reference samples and ratios to build as desired.

Heatmap:

1. Select samples for analysis in nSolver software
2. Select agglomerative clustering
3. Remove all non-endogenous probes
4. Remove probes with counts below 75

5. Select both Z-score transformations and Spearman distance calculation.

Correlation Analysis:

1. Select samples for analysis in nSolver software
2. Select Scatter plot
3. Select probes as above
4. In options, select: display R2 values and log axes.
5. Select probes to display and x axis probe.

Fold Change vs Expression Scatterplot

1. Export normalized data to excel
2. Average biological replicates for each condition
3. Take the log2 value of the ratio of the averages for the conditions to analyze
4. Plot the x axis as the log value of the "reference" counts
5. Plot the y axis as the value of calculated log 2 fold change.

Imaging of Fixed Hippocampal Sections:

IHC

Hippocampi were fixed in 4% paraformaldehyde overnight at 4^{o}C rotating in 1.5mL tubes. Hippocampi were then cryoprotected in 30% sucrose overnight at 4^{o}C. Treatment and control hippocampi were embedded in OCT side by side in order to match sections dorsoventrally. OCT was flash frozen on dry ice. 16uM sections were collected via cryostat on slides and washed 3 times with PBS (pH 7.4) for 10 minutes each. Sections

were then blocked and permeabilized for 2 hours in a humidified chamber at room temperature in 10% NGS, 0.3% Triton, PBS (10% NGST). Sections were then incubated in primary antibodies diluted in 1% NGST overnight at room temperature (Cell Signaling A177 Rabbit Lin28a 1:100, Millipore Mouse NeuN 1:200). Slides were washed 3 times in PBST for 10 minutes. Slides were then placed in TUNEL equilibration buffer for 10 minutes (TUNEL Dead End Fluorometric System from Promega). Slides were then treated with equilibration buffer with 8% TUNEL kit nucleotide mix and 2% TdT enzyme along with 1:1000 dilution of fluorescent secondary antibodies (Alexa 568 anti-rabbit Alexa 633 anti-mouse) for 1 hour in dark humid chamber at room temperature. Positive TUNEL controls were pre-treated with DNAse for 10 minutes and processed separately to avoid residual DNase activity as per manufacturer instructions. Negative controls were treated identically in the absence of TdT enzyme. Slides were placed in 2x SSC for 15 minutes to stop the TUNEL reaction, then washed 3 times with PBS at room temperature. 2 drops of DAPI were added to the top of each slide and incubated in the dark for 5 minutes (ThermoFisher NucBlue Fixed Cell imaging kit) Slides were covered in DAKO mounting media and coverslips secured with clear nailpolish. Stored protected from light at 4^{o}C.

Imaging

Stained sections were imaged with a 40x 1.3 NA, EC Plan Neofluoar on a Yokogawa spinning disk (Cell Observer, Carl Zeiss) and tiled to capture the whole hippocampal cross-section in the Zen software. 25 Z stacks were taken per tile for 3D analysis.

Nuclei Masking

Nuclei masking was done in Image J using maximum intensity projections of DAPI

staining. A threshold binary mask was created using the watershed tool. The mask was then applied to the TUNEL green channel image also binary thresholded and positive nuclei analyzed using Particle Analyzer.

NeuN/Lin28 Costain Analysis

Lin28a fluorescence in NeuN costained neurons was quantified in Imaris (BitPlane). ROIs were selected in CA2 and NeuN signal was used to make a mask using the Surface tool, with minimum sphere internally per surface set to 6uM. This mask was used to analyze Lin28a channel expression within NeuN marked neurons using average signal intensity within the mask, after subtracting average signal intensity within NeuN mask in sections not receiving Lin28a primary antibody but still stained with secondary. For molecular layer controls, a region between CA2 and the dentate gyrus with low NeuN signal was chosen, and Lin28a signal was assayed in a reverse NeuN mask to exclude any interneurons or blood vessel related nonspecific signal, with equivalent background subtraction via no primary controls.

RNA Isolation and RT qPCR

Total RNA from one half of each P13 hippocampal lysate was isolated in Tri-Reagent (Molecular Research Center, Inc.) according to the manufacturer's protocol. RNA pellets were air-dried and resuspended in 25 uL nuclease-free water. RNA concentration and quality were assayed by optical density (OD) at 260/280/230nm on a NanoDrop. (Ratios should be >1.8). 20ng of total isolated RNA was used for reverse transcription using candidate miRNA specific TaqMan (Applied Biosystems) primers in 15µl reactions according to manufacturer's instructions: 4^{o}C for 5 min, 16^{o}C for 30 min, 42^{o}C for 30 min, 85^{o}C for 5 min. CDNAs were then assayed via TaqMan miRNA assays for Let-7a

(000377), Let-7c (000379), and miR-132 (000457). The amount of u6 (002282) in each sample was used as a control to normalize all miRNA species. mRNA RT was performed using Applied Biosystems RNA to cDNA kit according to manufacturer's instructions. cDNA was assayed via TaqMan gene expression assay for Lin28B (Mm01190673_m1) and GAPDH (Mm99999915_g1) RT qPCR was performed on a Stratagene Mx3000P machine and accompanying software. Quantification was carried out using the standard-curve method.

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Publications

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Leadership & Activities

Leadership Initiative for the Environment (LIFE), VP 2011-2016

Incentive Mentoring Program, Tutor 2010-2012

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